The locust GABA receptor complex: The cage convulsant site

Brown, Michael Charles Simon

Award date:
1988

Awarding institution:
University of Bath

THE LOCUST GABA RECEPTOR COMPLEX :

THE CAGE CONVULSANT SITE.

submitted by; Michael Charles Simon Brown;

for the degree of PhD of the University of Bath.

1988

(signature).....

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

SUMMARY

A filtration assay was developed to measure the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS), to a washed membrane preparation from the isolated supraoesophageal ganglion of the locust, Schistocerca gregaria. The membranes exhibited high affinity, saturable binding of [^{35}S]TBPS, which was enhanced by the presence of chloride ions. The binding site shows positive cooperativity due to changes in on and off-rates with occupation by TBPS. The K_D was estimated to be 30 nM, although the $F_{0.5}$ was 400 nM. The B_{max} was 1200 fmoles/mg of membrane protein. The binding was enhanced by: GABA, benzodiazepines (Diazepam, Clonazepam & Ro5-4864) and pentobarbital, indicating that the binding site for TBPS is part of a GABA receptor complex. Insecticides which have been shown to give 'picrotoxinin-like' poisoning in mammals were also able to enhance the binding of TBPS, although picrotoxinin had no effect on the binding.

Parallel studies of [^{35}S]TBPS binding to rat brain membranes were able to reproduce previously reported Michaelis-Menten binding kinetics and inhibition of the binding by GABA and picrotoxinin.

Initial studies of ^{36}Cl flux into whole locust ganglia and membrane vesicles prepared from locust ganglia using two different preparations, were carried out.

CONTENTS

Figures

Tables

DEDICATION

I would like to dedicate this thesis to the memory of my father, who died before its completion. The endless support and encouragement which I have received from both my parents has enabled me to complete my education without the financial worries which have beset many of my peers. I am forever grateful.

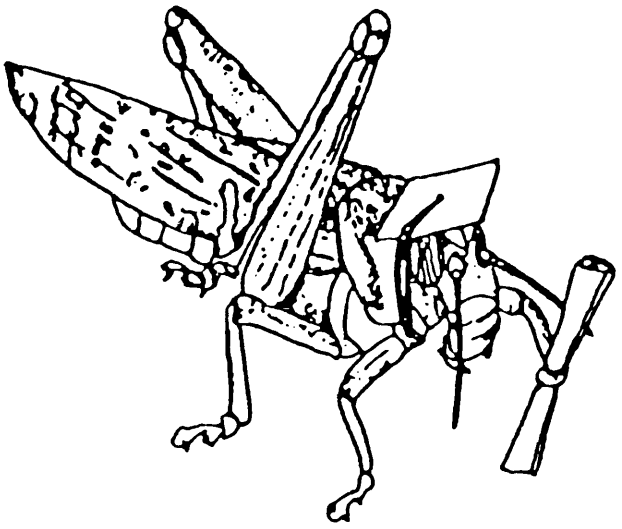
Also to Clare, who is my everything else.

ACKNOWLEDGEMENTS

I am grateful to the Dow Chemical Company for their financial support of the work presented here. Within Dow I would like to thank Mike Goosey and Andy Stapleton for their encouragement throughout the project.

I would like to thank all my colleagues and friends at Bath University for making my stay so rewarding. Especially to the locust team: Tim Robinson, Mike Duggan and Davie MacAllan for their stimulating discussions and Jo Ward for her art work and making the coffee.

Finally I would like to thank George Lunt for his advice and encouragement during my time at Bath.



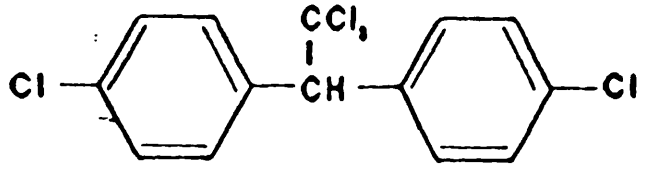
A LOCUST WAS HEARD TO COMPLAIN

"A BIOCHEMIST HAS DAMAGED MY BRAIN"

THE CAUSE OF HIS SORROW WAS

para-DICHLORO

DIPHENOL TRICHLOROETHANE.



$(C_6H_4Cl)_2CH.CCl_3$

ABBREVIATIONS USED

APS	3-aminopropanesulphonate
B	bound ligand
B_0	bound ligand at time zero
B_{eqm}	bound ligand at equilibrium
B_{max}	maximum number of binding sites
B_t	bound ligand at time, t
cpm	counts per minute
CNS	central nervous system
Ci	Curie
DDT	dichlorodiphenltrichloroethane
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulphonic acid
EC_{50}	concentration to give 50% enhancement (or effect)
EDTA	ethylene diamine tetracetic acid
EGTA	ethyleneglycolbis(B-aminoethylether-N,N,N',N', tetra acetic acid
$F_{0.5}$	concentration of ligand to give 0.5 occupancy
GABA	gamma-aminobutyric acid
GABA-T	4-aminobutyrate: 2-oxoglutarate aminotransferase
GAD	glutamic acid decarboxylase

IBP	isopropylbicyclophosphate
IC_{50}	concentration to give 50% inhibition
K_D	dissociation constant
K_I	inhibition constant
K_e	equilibrium constant of association at 0 occupancy
K_m	Michaelis rate constant
L	ligand
n_H	Hill coefficient
obs	observed
pH	percentage hydrogen ions
PMSF	phenylmethylsulphonylfluride
Ptx	picrotoxinin
R	receptor
RL	receptor ligand complex
RL_{eqm}	receptor ligand complex at equilibium
TBPS	t-butylbicyclophosphororthionate
Tris	tris[hydroxymethyl]-aminomethane

Chapter 1: General Introduction

1.1 The Desert Locust, Schistocerca gregaria

1.1.1 An age old adversary

"Moses stretched forth his rod over the land of Egypt, and the Lord brought an east wind upon that land all that day and all that night; and when it was morning the east wind had brought the locusts. And the locusts came up over the whole land of Egypt, and settled on the whole country of Egypt, such a dense swarm of locusts as had never been before or ever shall be again. For they covered the face of the whole land, so that the land was darkened, and they ate all the plants of the land and the fruit of all the trees which the hail had left; not a green thing remained, neither tree nor plant of the field, through all the land of Egypt." Exodus 10 v13-16 (Moses c1290 B.C.)

Among the most ancient and potentially the most widespread serious pest of standing crops are locusts. There are numerous mentions of locusts in the bible and one of the versions of the Koran describes them as "the teeth of the wind". These references are always full of disaster and destruction on unbelievable scales, which we now know from modern records is no exaggeration. During the plague that continued from 1948 - 1963, several swarms were recorded as exceeding 100 square miles and one is said to have been the size of London. Additionally, contrary to common belief, outbreaks of

many species of locusts and grasshoppers become not less severe but more so as development of mankind increases. Due to locusts excessive multiplication and spread, the formation of areas suitable to outbreaks are furthered, not hindered, by extended and improved cultivation. Consequently the locust problem has increased over the last half century, and this has resulted in vast, coordinated international cooperation in an attempt to predict and control locust plagues.

1.1.2 Life cycle

The outstanding biological characteristic of locusts is their ability to respond to changes in population density behaviourally, physiologically and morphologically. Individuals occurring in low-density populations typically behave like grasshoppers and belong to the solitarious phase. When crowded together they undergo a transformation to the gregarious phase and form persistent groups of nymphs (hopper bands) or adults (swarms). The opposite of this process of gregarisation is dissociation. Intermediate forms belong to the transient phase. The theory of phase polymorphism was first advanced by Uvarov (1921) for Locusta migratoria and has been since shown to apply to other species.

The outstanding differences between phases are behavioural. Gregarious hoppers form cohesive marching bands. The word conjures up a picture of companies, regiments, whole armies on the move. This in a sense, is what they may well be as one small group of hoppers, a day or two old, links up with another, or a band already fairly large, joins perhaps with another one larger yet. At its maximum stage of expansion such a band may be 10 miles wide and many more long and will march from dawn to dusk or later but always with small groups or individuals within it coming to temporary halts, to feed, to rest, to bask in the sun, yet always to join the forward movement again. The more its numbers, the more it marches. The bigger the crowds, the bigger the mutual stimulus.

Solitarious hoppers move little and avoid one another until they become habituated to their fellows (Ellis 1959).

Gregarious adults form cohesive swarms which characteristically fly by day, and extend the distribution area of the species during plagues. Solitarious and transient adults fly mainly by night, when lower temperatures restrict their distribution.

Surprisingly no locust is born with the ability to group but must acquire it afresh in every generation. This is simple enough for the progeny of parents which have

already become gregarious. Eggs are laid in groups and from the moment of hatching the young hoppers find themselves so crowded that they are conditioned into gregarious behaviour very quickly. Solitaria hoppers may also learn to group under various circumstances. The habitat in which they normally live their quiet unharmful lives may be invaded by swarms or hopper bands. Contracting vegetation as the always-brief greenery of the wadis has withered in the presence of drought results in crowding. Contact will then enforce the learning of gregarious ways and if continued will produce a complete phase change.

During recessions most reports refer to hoppers and adults at low densities with occasional reports of bands and swarms. Recession populations migrate within the recession area, moving with the wind (Rao 1942) between areas of seasonal or erratic rainfall,in which they breed, and where outbreaks sometimes occur (Waloff 1966).

The occurrence of these breeding and outbreak areas depends on variable weather systems which transport locusts and bring rain. However, the incidence of outbreak conditions has been repeatedly associated with areas of marked relief in desert regions, or with areas where flying locusts may be concentrated by

convergent winds which occur in the tropics (Waloff 1972).

1.1.3 Natural enemies of the Desert Locust

During recession periods the natural enemies of the Desert Locust play a part in controlling the outbreak of swarms. Although these enemies are not numerous enough to control a plague, however, if they were not present, man's efforts to control the Desert Locust would need to be even more vigorous.

The insect enemies of the Desert Locust can be crudely divided into two groups:

1) Egg parasites;

Several parasitic wasps of the genus Scelio destroy the eggs of locusts by laying their own eggs in the same egg pod which hatch earlier and feed on the locust eggs. Additionally flies such as Stomorhina lunata and Systoechus somali attack locust egg pods in a similar manner.

2) Parasites of hoppers and adults;

Blaesoxipha filipjevi do not lay eggs. The eggs hatch inside the female's body. When the female contains larvae it becomes very active, and if a locust flies by it darts after it and strikes it. The locust falls to the ground with a newly born fly larvae on the underside of its wing. The larvae then burrows into the body of

the locust. The larvae of the fly Symmictus costatus hatch from eggs laid in cracks in the soil, or the bark of trees. They are blown about by the wind and when they encounter hopper locusts, enter them through the soft membrane of the hoppers until they are ready to pupate.

1.1.4 Control of the Desert Locust

The life cycle of Schistocerca gregaria is geared to survival in an environment of extreme hostility rather than, as might be thought, to plunder in an easy one. Thus the problem of preventing plagues of the desert locust has proved to be complex. The main difficulties arising from:

1) the great mobility of Desert Locust adults in all phases. There is no single place in the whole of the desert breeding areas of over 5 million square miles where it may be sure of finding its needed habitat. This means that the Desert Locust must have extraordinary powers of flight to be able to traverse the vast distances required to reach where rains have fallen. The insect's flight fuel is fat and its store can keep it in the air for spells of up to 17 hours. Thus in terms of muscular activity in proportion to body size, a flying locust works 10 - 20 times as hard as a human being running at top speed;

2) the very unstable environment in the recession area;

3) the resultant continual fluctuations in numbers, and density and phase of recession populations;

4) the large size of the recession area and the emptiness and geographical remoteness of much of the terrain in which outbreaks and plague upsurges occur (Hemming et al.,1979).

There are a number of approaches which have been suggested or attempted:

a) Ecological control.

The ability of the Desert Locust to move on to a more suitable area severely limits the possibility of controlling it by ecological means. Flooding has been tried (Gunn 1960) which controlled the locusts in the area but other locusts was still able to migrate into nearby areas and breed.

b) Physical methods.

The old mechanical methods of driving hoppers into trenches, beating them, or collecting with machines, have all been failures and are also inadequate for the magnitude of the problem. (Gunn 1979).

c) Insecticide methods.

From 1880 to the 1950's, locust hoppers of several species were attacked using moistened wheat bran containing an arsenical, a stomach poison. This had the disadvantage that the bait was not taken after it had

dried up, which it did within a few hours in hot sun, thus the insecticide did not reach its target. With the advent of lindane, (a potent contact insecticide) available as a fine powder, the bait could be used dry. However the disadvantage for control of the desert locust was the very large tonnages of bait required to attempt to control an outbreak (Gunn, 1979).

With the arrival of insecticides which did not harm the plants such as dieldrin there was a return to applying the insecticide to the standing vegetation "vegetation baiting" (Gunn 1952).

This could be done using two methods:

i) Exhaust-nozzle spraying.

A device was fitted to the exhaust of a Land Rover which produced a fine spray of the insecticide. The poison was then emitted downwind as the Land Rover drove square across the wind. This made a wide band of vegetation toxic to insects. Another such band would then be laid upwind, parallel with the first, at a distance equal to the distance the hoppers were likely to walk (Sayer 1959).

ii) Aircraft spraying.

This technique is basically similar to Sayer's, except the strips of insecticide were laid down by an aircraft instead of a ground vehicle and cross strips were also

put down making a lattice. (Mallamaire & Roy 1958). Hundreds of square kilometres could be treated quickly and economically using this method and it could also gain access to areas where ground vehicles could not go.

1.1.5 The future

The availability of such techniques has led to massive use of insecticides such as dieldrin and DDT over large areas to try to keep the locusts in their solitarious phase. However indiscriminate use of insecticides allows the spectre of chemical resistance in the locust to enter on the scene. It was because of the possibility of locusts developing resistance that experiments in blanket spraying in Saudi Arabia were viewed with dismay by some biologists. When one realises that insecticidal methods have been the only successful approach to control of the desert locust, resistance in this species would indeed be a very serious problem.

Another objection to blanket spraying, is that when non-specific chemicals like dieldrin and DDT are used, they knock out not only the target pests but also the parasites and some of the predators (small birds) which are nature's means of control. Additionally when such spraying is carried out near settlements, valuable livestock may be lost through contamination of food and water, making the effects of the locust attack even worse for the local population.

Clearly if insecticidal methods are to remain our primary weapon against the desert locust, a locust specific insecticide, which was harmless to the locust's parasites, predators and to the region's livestock would be a great advantage. Then if resistance does develop one would not have interfered with nature's own damage control mechanism and total disaster may be avoided.

1.2 The Insect Central Nervous System

1.2.1 General organisation

In the central nervous system of the insect, perikarya of motor and interneurons are aggregated to form ganglia within which they are grouped peripherally. Fig.1, shows a transverse section of an abdominal ganglion.

Each neuron is almost completely wrapped in one or more glial cells. The glial cells form an insulating, protective sheath around the neuron. The glial cells are thought to insulate axons from each other, limiting the number of synaptic connections a neuron can make. It is believed that synapses only occur where glial folds are absent. The perikarya of the neurons are situated near the periphery of the ganglion, close beneath the perineurium, the first layer of cells inside the neural lamella.

The centres of the ganglia are occupied by the neuropile, a complex of afferent, interneuron and efferent fibres and their supporting glial elements. Within the neuropile groups of fibres may be similarily orientated so as to form fibre tracts. There are no cell bodies in the neuropile. The glial material is reduced in the body of the neuropile and may be absent altogether, allowing adjacent axons to be in contact

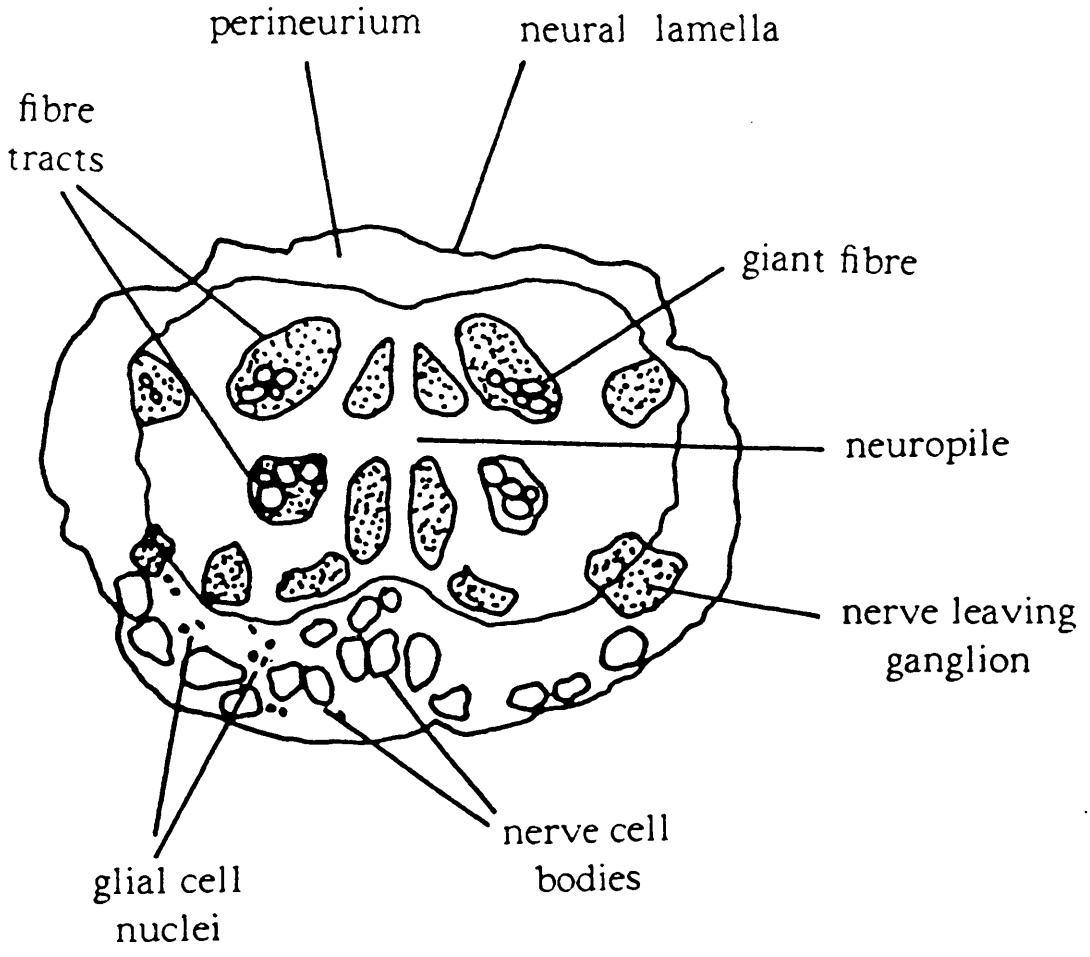


Figure 1. Transverse section of an abdominal ganglion

with each other . It is believed that most synapses occur in the neuropile.

The most anterior ganglion is the brain or cerebral ganglion, lying dorsal to the oesophagus in the head. From the brain the circumoesophageal connectives pass, one either side of the oesophagus, to the first of a chain of ganglia lying ventrally. The ganglia are joined to each other longitudinally by connectives made up only of axons and supporting cells, while extending from each ganglion to the peripheral sense organs and effectors are the peripheral nerves. These are usually aggregations of both motor and sensory fibres.

1.2.2 The brain

The brain is the principal association centre of the body, receiving sensory input from sense organs of the head and, via ascending interneurons from the more posterior ganglia. Motor output from the brain supplies the antennal muscles and passes via descending premotor interneuronal fibres to the posterior ganglia, controlling the activities of the rest of the nervous system to some extent. It is also the source of many long-term organised behaviour patterns and governs their modification by learning.

The brain can be divided up into three regions: the protocerebrum, deutocerebrum and tritocerebrum (see fig. 2).

Protocerebrum.

The protocerebrum is bilobed and is continuous laterally with the optic lobes. It is the most complex part of the brain.

In the centre of the protocerebrum a mass of neuropile forms the central body to which axons converge from many parts of the brain. It is thought to be a major source of premotor outflow from the brain to the ventral nerve cord.

Deutocerebrum.

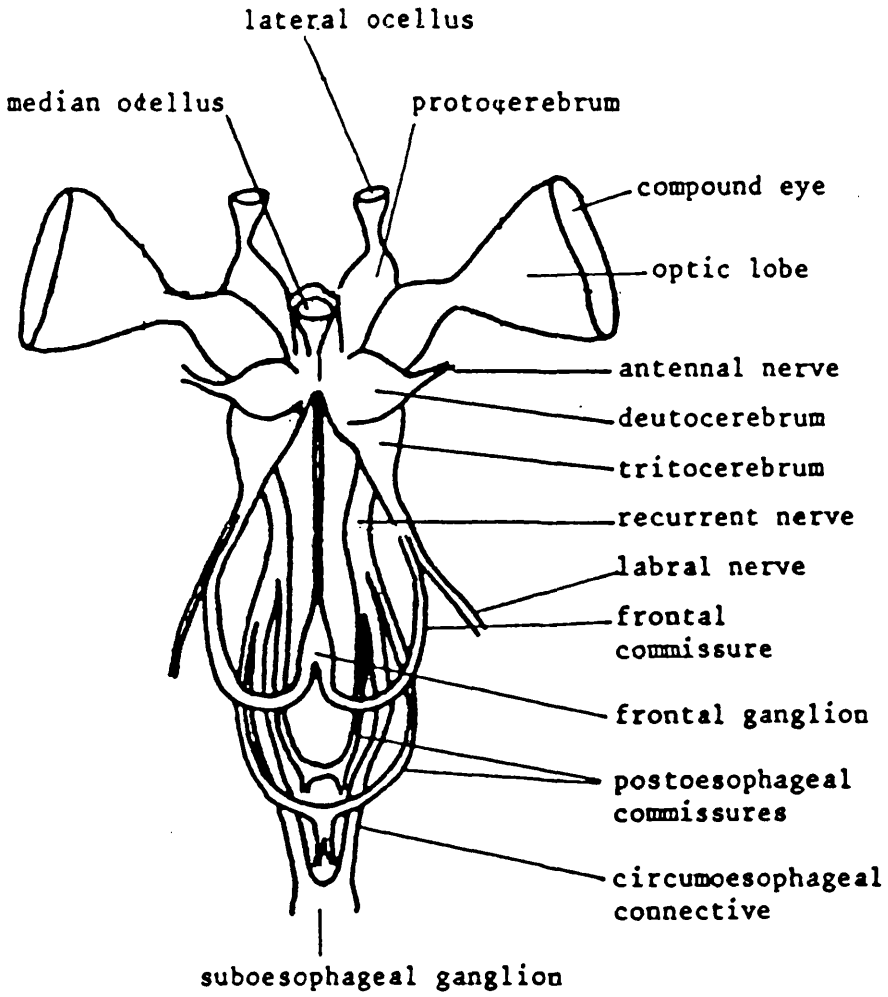
The deutocerebrum contains the antennal lobes which are divided into dorsal sensory and ventral motor areas. As are the antennal nerves which enter this part of the brain and contain both sensory and motor elements.

Tritocerebrum.

This is a small part of the brain consisting of a pair of lobes beneath the deutocerebrum. Circumoesophageal connectives pass from the tritocerebrum to the suboesophageal ganglion.

For further detail one should consult "The Insects : Structure & Function" R.F. Chapman (1975).

Figure 2. View of the brain of a locust



The brain of the locust was the nervous tissue which was used for the studies described in this thesis.

The detailed knowledge of the invertebrate nervous system stems partially from its simplicity, and the ease with which it can be studied in detail. This contrasts with the limited information available on the detailed physiology of vertebrate nervous systems due to the extreme complexity of these systems.

Consequently insects have often been the animals of choice for electrophysiological studies.

However when one turns to neurochemistry the case is reversed. We know extremely little about the chemical events involved in invertebrate neurotransmission. This lack of knowledge is one aspect of the general problem posed by the very small quantities of nervous tissue available to the invertebrate neurochemist.

But if such serious pests as the Desert Locust are to continue to be successfully controlled by insecticides, one needs to fill this gap in our knowledge concerning chemical neurotransmission in locusts and insects in general. Such information will be of importance because many current insecticides affect the nervous system, (see 1.3.3). Thus if one is to develop better, more specific insecticides one needs a

thorough knowledge of neurotransmission, both electrophysiologically and neurochemically.

This is one of the reasons that our laboratory has been studying the neurochemistry of the Desert Locust for a number of years. A second reason is that the Desert Locust is one of the largest pest insects available, which reduces to a small extent, the problem of the small quantities of nervous tissue available for such studies.

1.3 The Pesticide Industry

1.3.1 The Economic Importance of Insects

1.1 illustrated how the adaptation of the Desert Locust to its extremely harsh environment has made it a very formidable adversary to man's efforts at cultivation of the land. However in the broad sense of their function in the cycle of nature, insects are in fact beneficial to man. Compared with the total number of insect species (there are over 800 000 species comprising the phylum Arthropoda, of which the insects are by far the major constituents), the pests are few but their effect is immense.

A small number of insects have obvious value, such as the predators and parasites of pest insects, termites which are used for food in Africa, and some beetles and Lepidoptera which are used in making articles of jewellery and ornaments. However the greatest value of all sprang from our sweet tooth and liking for honey!

The honey bee is the supreme pollinator and this action has been of more value than the honey that they make, especially as human population has increased and it has become necessary to produce more food per acre of ground. (Exactly the same set of circumstances which make the Desert Locust more likely to swarm!)

The fruit tree industry all over the world is dependent on the honey bee, whose highly sophisticated social organisation ensures the necessary numbers of pollinators. Although most field crops do not depend on the honey bee, being self or wind pollinated they do benefit from the widespread use of nitrogen fixing legumes.

Thus, it is ironic that modern large scale farming with its widespread and often indiscriminate use of insecticides should have contributed to the destruction of honey bees as well as inducing resistance in pest insects. This realisation has prompted the pesticide industry to become more interested in basic research into insect species which may enable them to develop insect specific insecticides and to avoid the problems of resistance.

1.3.2 Pesticides in history

The idea of combatting pests by the use of chemicals is not new; sulphur was known to avert insect pests before 1000 B.C. However it was in the 16th century when the Chinese were using moderate amounts of arsenic as an insecticide, that the seeds of an industry were sown. Then in the 17th century the first naturally occurring insecticide, nicotine - from extracts of tobacco leaves - was used to control the plum curculio and the lace bug. These early discoveries were probably

a combination of careful observation following trial and error. This was followed in 1850 by the discovery of two natural insecticides, which proved to be so successful that they are still in use today; rotenone from the roots of the derris plant and pyrethrum from the flower heads of a species of chrysanthemum.

Additionally most of the well known poisons have been applied at one time or another for the control of insects, but often proved hazardous to the operators. For example, from 1886 in California hydrogen cyanide gas was used against scale insects on citrus trees. Tents would be placed over the trees and hydrogen cyanide generated inside. Initially this treatment proved to be a success but after continued use failures were increasingly found. This was due to the development of resistant strains of insect. This was the first reported example of resistance to an insecticide.

However it was not until 1930 that the modern era of synthetic insecticides began with the introduction of alkyl thiocyanate and then in 1939 Dr. Paul Muller discovered the powerful insecticidal properties of dichlorodiphenyltrichloroethane or DDT. Following successful field tests this was manufactured in 1943 and rapidly became the single most widely used insecticide in the world. Following on from the success of DDT were several insecticidal analogues and other types of

organochlorine compounds which were also potent contact insecticides; hexachlorocyclohexane (or lindane) and several chlorinated hydrocarbon cyclodiene compounds, such as aldrin, dieldrin, heptachlor and endrin. However these did not reach widespread use until the middle 1950's.

The organophosphorus compounds represent another extremely important class of insecticides. Their early development stemmed from wartime research on nerve gases for use in chemical warfare by Dr Gerhard Schrader. Unfortunately such compounds were highly poisonous to mammals and later research in this field has been increasingly directed towards the discovery of more selective and less poisonous insecticides.

A closely related group of insecticides are the carbamates, first discovered by the Geigy company in Switzerland in 1947. These are increasingly being used to replace DDT.

1.3.3 Classes of insecticides & their actions

1.3.3.1 Botanical insecticides

a) Nicotine: As early as 1690 water extracts of tobacco leaves were being used to kill sucking insects on garden plants (Jacobson & Crosby 1971). The active principle in tobacco extracts was isolated in 1828 and shown to be the alkaloid nicotine. The structure was not elucidated until 1893 (Martin 1973).

Nicotine functions as a non-persistent contact insecticide against aphids, capsids, leaf miner, codling moth and thrips, on a wide variety of crops. However its use is declining as it is replaced by synthetic insecticides and due to its high vertebrate toxicity.

Nicotine kills vertebrates because it mimics acetylcholine at the neuromuscular junction causing twitching, convulsions and finally death. Nicotine probably has a similar mode of action in the insect CNS. However there is no cholinergic innervation of insect skeletal muscles.

b) Rotenoids: These are a group of insecticidal compounds occurring in the roots of Derris elliptica, from the East Indies and Malaya, and a species of Lonchocarpus from South America.

Rotenoids are toxic to fish and many insects, but are almost harmless to most warm-blooded animals. Derris was

widely used in cattle and sheep dips for the control of ticks and other ectoparasites, but has recently been superseded by synthetic insecticides. Now it is primarily used in horticulture against aphids, caterpillars, sawflies, wasps raspberry beetles and red spider.

The biochemical mode of insecticidal action appears to involve the inhibition of mitochondrial electron transport (Corbett 1974).

c) Pyrethroids: Pyrethrum is a contact insecticide obtained from the flower heads of Chrysanthemum cinerariaefolium and has been used as an insecticide since ancient times. Pyrethrum owes its importance to the outstanding rapid knockdown action of a few seconds, on flying insects combined with a very low mammalian toxicity, due to its ready metabolism to non-toxic products.

Pyrethrum contains four main insecticidal components which are collectively termed pyrethrins. The synthesis of the pyrethrins has resulted in the production of synthetic pyrethroids, the first of which was allethrin. The synthetic pyrethroids are now the most effective insecticides on the market.

Pyrethroids act on the voltage dependent Na^+ channels in neuronal axons. They hold open these channels causing

chronic firing of the nerves, which results in convulsions in the insect.

1.3.3.2 Synthetic insecticides

a) Organochlorines: The most important member of this group of insecticides is 1,1,1-trichloro-2,2-di-(p-chlorophenyl)ethane or DDT. DDT is thought to act at the same site as the pyrethroids.

b) Hexachlorocyclohexane: Was discovered by ICI in Britain in 1942. Hexachlorocyclohexane can exist as eight different stereoisomers, five of which are found in the crude product. Only the gamma isomer or lindane has powerful insecticidal properties. As a spray lindane is useful against many sucking and biting pests, and as smoke for control of pests in grain stores.

Originally lindane was thought to act at the same target as DDT but recent work has suggested that it may well interact at the GABA receptor complex, (see section 2.3.1.2).

c) Cyclodienes: The first of this new group of organochlorine insecticides, chlorodane, was discovered in 1945. They are prepared from hexachloropentadiene by the Diels-Alder reaction. This is the origin of the names for the two best known members of the group, Aldrin and Dieldrin. Aldrin, dieldrin and endrin are some of the most active general contact insecticides and

like lindane they appear to act at the GABA receptor complex, (see 2.3.1.2).

d) Organophosphorus compounds: Investigation into the synthesis of toxic organophosphorus compounds as potential nerve gases began during the Second World War. All the nerve gases discovered were powerful insecticides but they have never been extensively used due to their high mammalian toxicity. Thus further research was directed towards synthesising more selective organophosphorus compounds. Various companies are marketing numerous organophosphorus compounds which have relatively low mammalian toxicity but which share the same mode of action in insects.

The insecticidal organophosphorus compounds apparently inhibit the action of many enzymes, but their most profound effect is the inhibition of acetylcholinesterase. This enzyme terminates the action of acetylcholine at its synapse by hydrolysing the released acetylcholine. Thus inhibition of acetylcholinesterase results in chronic stimulation of the postsynaptic cell by acetylcholine and at the mammalian neuromuscluar junction results in paralysis, a similar mode of action may occur in insect CNS (Corbett 1974).

e) Carbamates: The successful development of organophosphorus insecticides stimulated investigation

of other compounds known to have anticholinesterase activity. One such compound is the alkaloid physostigmine, the active ingredient in calaban beans. The physiological properties of this alkaloid were supposed to be based on the phenylmethylcarbamate part of the structure. Investigation of similar compounds led to the discovery of a number of parasympathomimetic drugs like neostigmine. However recent work has indicated that carbamates are able to open the acetylcholine gated ion channel at much lower concentrations than are required to inhibit acetylcholinesterase (Alberquerque et al., 1988). This work suggests that a major mechanism for the action of carbamates may well be as nicotinic agonists.

1.3.4 Future developments

The ideal chemical pesticide would have high specific toxicity against the target pest, should not persist longer than necessary to achieve its objective and would not affect the rest of the ecosystem, so that natural predators and other beneficial insects are unharmed. However the majority of pesticides currently in use fall far short of these ideals.

Thus it is important that one continues to carry out basic research into the mode of action of current insecticides, in order that one may learn from one's mistakes or find new directions for their development

Eventually one may have sufficient data to rationally design tomorrows insecticides to meet the ideal.

1.4 Summary

If one is to continue to control the Desert Locust by chemical means, one needs to obtain a thorough understanding of potential target systems in insects, and of the mode of action of existing and novel insecticides in invertebrates and vertebrates. In this way the ideals of high specific toxicity to pests and not the natural predators and other beneficial insects can be achieved.

The next chapter will set out why the GABA receptor complex is one such potential target site which needs continued basic research in pest species.

Chapter 2 : GABAergic neurotransmission

2.1 Historical Aspects

gamma-aminobutyric acid (GABA) was discovered at the beginning of the century (Ackerman & Kutsher, 1910), but it was not until 1950 that it was demonstrated to be present in mammalian brain (Awapara et al., 1950; Roberts & Frankel, 1950; Udenfriend, 1950). Following some controversy over the role of GABA as a neurotransmitter, Kravitz demonstrated GABA to be present in lobster inhibitory neurones, where it is synthesised, accumulated and released (Kravitz, 1967). By the early 1970s it was thought that GABA might be the universal transmitter of junctional neuromuscular inhibition in all invertebrate phyla, from nematodes to arthropods, and also to be involved in the CNS of crustaceans, insects and possibly molluscs (Gerschenfeld, 1973; Pichon, 1974; Callec, 1974). However the evidence for this was virtually all electrophysiological, and despite the early studies on GABA involving invertebrate tissues, the bulk of the biochemistry of the GABAergic neurotransmission has been elucidated in mammalian tissues.

2.2 Mammalian GABA System

There have been several detailed reviews of the now well characterised mammalian GABA system (eg Olsen, 1981; Turner & Whittle, 1983; Bradford, 1986). This is schematically summarised in fig. 3.

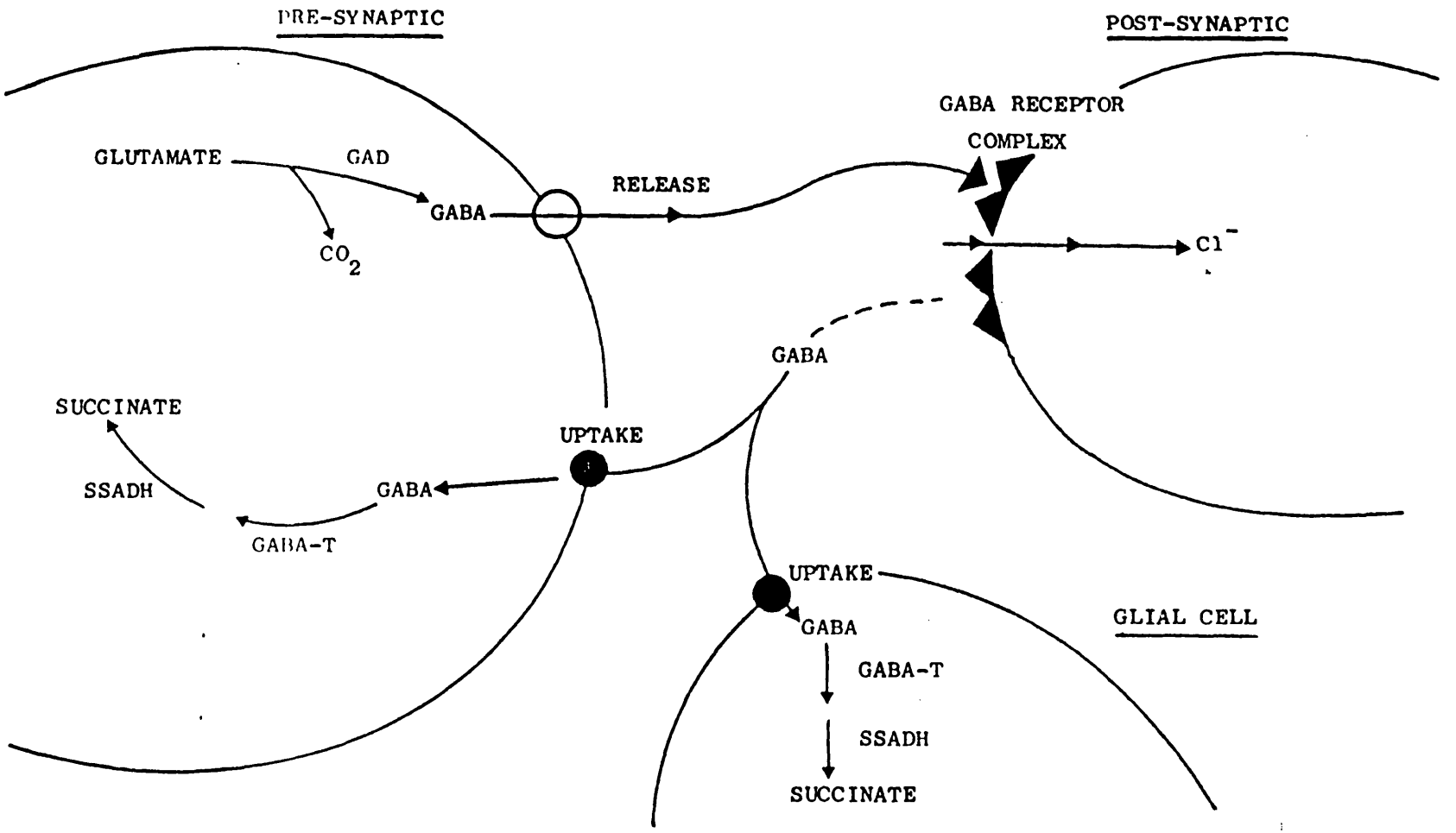


Figure 3. Schematic representation of a GABAergic synapse

Additionally the protein chemistry of the solubilised receptor has recently culminated in the cloning of the mammalian GABA receptor by the groups of Barnard & Seeburg. This work and that leading up to it has been reviewed by Stephenson (1988).

2.2.1 GABA synthesis

GABA is biosynthesised by the important pyridoxal phosphate-dependent enzyme, glutamic acid decarboxylase (GAD; EC 4.1.1.15). This acts on glutamate and removes the gamma-carboxyl group as CO_2, to produce a gamma-amino acid. The glutamate is readily produced via the citric acid cycle. GAD appears to be localised to the nerve terminals of neurons utilising GABA.

2.2.2 Release and inactivation of GABA

Studies of GABA release in vivo from cerebral cortex or substantia nigra, in response to depolarising chemical stimuli or to neural pathway activation, provide clear supporting evidence for a transmitter role for GABA in these brain regions (Bradford, 1986).

Initial functional inactivation of GABA is by a transport system displaying high affinity and low K_m value for GABA. The transport system is located specifically in the neurons releasing GABA and in surrounding glial cells.

Once taken up, metabolic conversion or re-use follows. GABA is absorbed into glial-cell or neuronal mitochondria, where it becomes transaminated to succinic semialdehyde by GABA-alpha-oxoglutarate transaminase (EC 2.6.1.19), or GABA transaminase (GABA-T). Succinic semialdehyde in turn, is oxidised to succinic acid by succinate semialdehyde dehydrogenase, and enters the citric acid cycle of the glial cell.

Thus, a whole tissue sample, such as a cortex homogenate, displays the ability to synthesise GABA from glutamate and subsequently transaminate it and oxidise the product. A sequence known as the GABA shunt pathway (see fig. 4).

2.2.3 Physiological action of GABA

When GABA is released from a nerve terminal the most common post-synaptic event involves a rapid and transient increase in membrane permeability to chloride ions (Cl^-), which results in a hyperpolarisation of the post-synaptic cell (Nistri et al.,1980). This change in Cl^- permeability is brought about by the interaction of GABA with a post-synaptic membrane receptor (Enna & Snyder, 1975). This physiological change can be blocked by the antagonist, bicuculline, or by the plant alkaloid picrotoxinin, which inhibits the increase in Cl^- conductance mediated by GABA. The bicuculline-sensitive sites for GABA have been extensively studied in kinetic

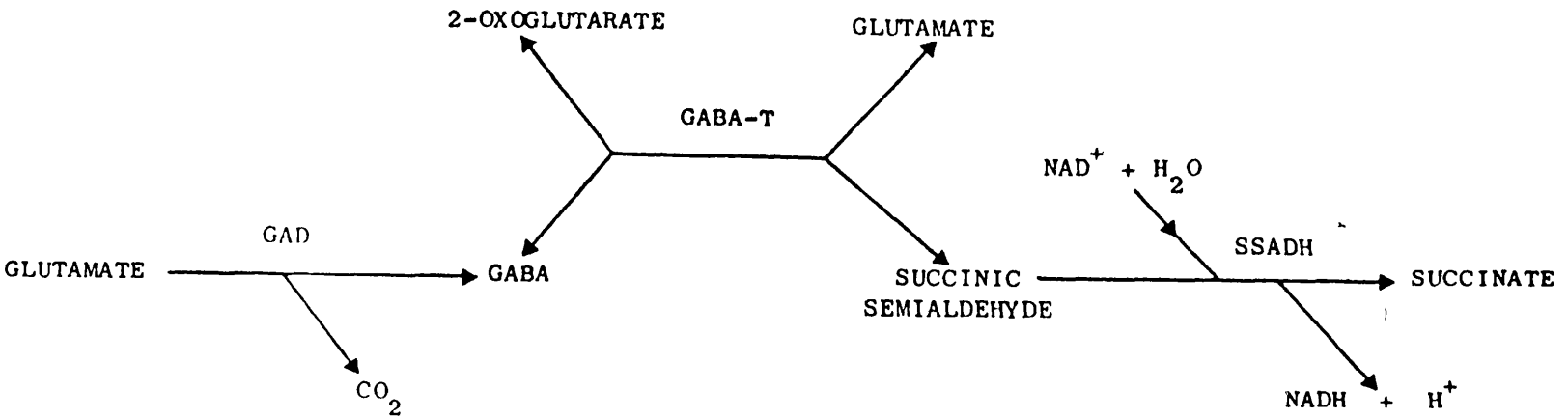


Figure 4. The "GABA Shunt": metabolic pathway of GABA in mammalian nervous tissue

and molecular terms and have been designated $GABA_A$ receptors. The GABA binding site is not only coupled to the Cl^- channel, but is also allosterically modulated by benzodiazepines and barbiturates.

$GABA_B$ receptors appear to be pre-synaptically located and involved in reduction of neurotransmitter release. $GABA_B$ receptors are sensitive to the agonist Baclofen, which is ineffective at $GABA_A$ receptors. The $GABA_A$ antagonists, bicuculline and picrotoxinin are unable to antagonise the action of baclofen on $GABA_B$ receptors. Although the mode of action of $GABA_B$ receptors has not been studied as thoroughly as that for the $GABA_A$ receptors, it appears to involve modulation of Ca^{2+} channels and interaction with guanine nucleotide binding proteins. Thus its mode of action may well involve the modification of cyclic-AMP levels in the cell (Bradford, 1986).

This introduction to mammalian GABA neurotransmission will concentrate on the $GABA_A$ receptor. This is because the work in this thesis concerns the GABA receptor in the CNS of the Desert Locust which appears to be a $GABA_A$ type receptor (Robinson et al., 1986).

2.2.4 Pharmacology of the $GABA_A$ receptor

2.2.4.1 GABA binding site

The GABA binding site is usually quantified using tritiated GABA or its agonist muscimol. Other potent agonists are 3-aminopropanesulphonate (APS) and isoguvacine. As mentioned above bicuculline is a potent, competitive antagonist at this site. Picrotoxinin is a noncompetitive antagonist of GABA binding, (see 2.2.4.4). When binding is carried out in sodium free buffers (to avoid GABA binding to uptake sites) on freeze/thawed and extensively washed brain membranes, or detergent treated membranes (to wash out endogenous GABA), two binding sites are observed for GABA. Only the low affinity site:

K_D = 300 nM and

B_{max} = 1-2 pmole/mg membrane protein

(Enna and Snyder , 1975) was observed in untreated membranes. In treated membranes an additional site:

K_D = 20 nM and

B_{max} = 0.5 pmole/mg membrane protein

was observed (Olsen, 1980).

2.2.4.2 Benzodiazepine site

The benzodiazepines are the most widely used tranquilisers in clinical practice. They are very good

at relieving anxiety and can be used as anticonvulsants and muscle relaxants. Diazepam or "Valium" is the prototype compound. Tritiated diazepam binds to high-affinity sites:

K_D = 4 nM and,

B_{max} = 1 pmole/mg membrane protein,

in mammalian brain. Such binding shows a chemical specificity correlating with the activity of a series of benzodiazepines as CNS depressant agents with anxiolytic, anticonvulsant, sedative-hypnotic and muscle relaxant actions, (Tallman et al., 1980).

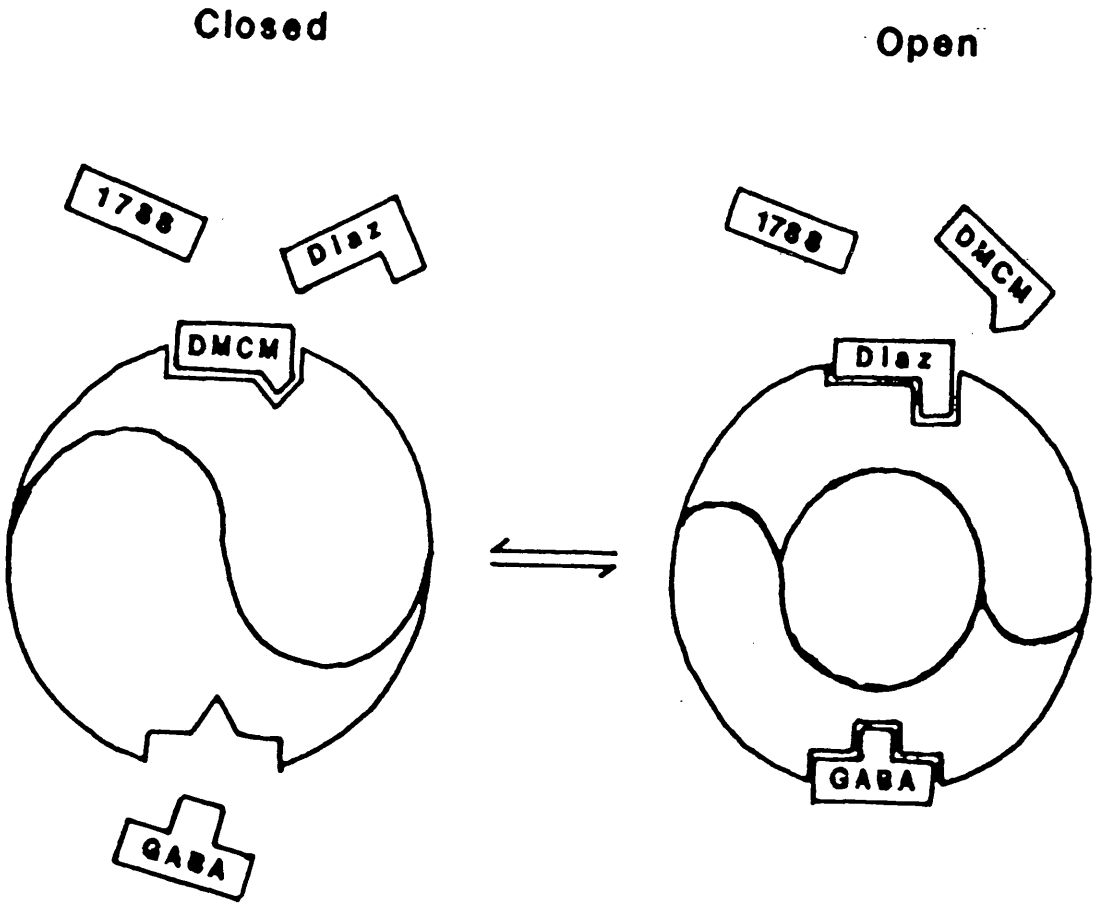
The binding of benzodiazepines can be enhanced by GABA and its agonists. Bicuculline reverses the GABA enhancement and reduces baseline benzodiazepine binding. Numerous workers have been able to demonstrate this effect (see Olsen, 1981). However the reverse is not true. Most groups have been unable to demonstrate any effect of benzodiazepines on GABA binding. Only Guidotti's group have been able to observe an enhancement of GABA binding by benzodiazepines (Guidotti et al., 1978). Electrophysiological studies have shown that in the presence of GABA the benzodiazepines cause an increase in the frequency of chloride channel opening, thus facilitating and increasing the efficiency of GABA transmission (Study & Barker, 1981). Such observations suggest a non-competitive but allosteric

interaction between some of the GABA binding sites and some benzodiazepine binding sites.

2.2.4.2.1 "Inverse agonists" & cooperativity at benzodiazepine receptors

There are other drugs which have been shown to interact at the benzodiazepine binding site and indicate further complexity of the GABA receptor complex. Among these compounds is the imidazodiazepine, Ro15-1788, which has no clinical activity alone but potently antagonises the actions of anticonvulsant benzodiazepines, such as diazepam (Bonetti et al. 1982). Also some esters of beta-carboline-3-carboxlic acid, which produce convulsions when administered alone, appear to act at the benzodiazepine binding site (Braestrup et al. 1982). Because the beta-carbolines on their own produce effects which oppose the action of benzodiazepines, they have been termed 'inverse agonists'. Both the anticonvulsant action of benzodiazepine and the convulsant action of the beta-carbolines are antagonised by Ro15-1788 (Braestrup et al. 1982). The actions of agonist benzodiazepines, the antagonist, Ro15-1788 and the inverse agonist beta-carbolines have been explained by Ehlert (1986), who has proposed a two-state model for the GABA receptor complex, (see fig. 5).

Figure5. Ehlert's Two-state model for the GABA receptor complex



GABA = GABA binding site agonist
Diaz = Diazepam, benzodazepine agonist
DMCM = βcarboline, benzodiazepine inverse agonist
1788 = Ro15-1788, benzodiazepine antagonist

He proposes that GABA binds selectively to the open state of the chloride channel complex causing the channel to remain open and allowing a flux of chloride ions. A benzodiazepine like diazepam, enhances the binding of GABA by also being selective for the open state of the receptor channel complex. The interaction between GABA and diazepam is called positive heterotropic cooperativity. Thus the convulsant action of beta-carbolines can be rationalised by assuming that they have selectivity for the closed state of the receptor channel complex and thus allosterically inhibit the binding of GABA. This type of interaction is called negative heterotropic cooperativity. Ligands for the benzodiazepine binding site which have equal affinity for ground and active states of the receptor channel complex should display no pharmacological activity of their own. However they should block both the negative and positive heterotropic effects of other ligands, which bind to the benzodiazepine binding site. Ro15-1788 demonstrates these characteristics.

2.2.4.2.2 Heterogeneity of benzodiazepine binding sites

Such benzodiazepine binding sites present in the CNS were designated 'central' after the demonstration of [^{3}H]flunitrazepam binding on rat kidney membranes. Those benzodiazepine binding sites in rat kidney membranes

were thus designated 'peripheral' (Regan et al. 1981). The central and peripheral binding sites can be distinguished by their pharmacology. Central binding sites are sensitive to clonazepam, whilst peripheral binding sites are sensitive to Ro5-4864. The availability of [^{3}H]Ro5-4864 led to the discovery of peripheral type binding sites in the CNS (Schoemaker et al., 1981). However the binding of Ro5-4864 to brain peripheral binding sites is not affected by GABA and the sites cannot be photoaffinity labelled by flunitrazepam, unlike the central binding sites (Thomas & Tallman, 1981). There is still much debate as to exactly where these brain peripheral benzodiazepine binding sites are located, in the brain and within the cell and also about the function of these binding sites.

2.2.4.3 Barbiturate site

Other classes of drugs are able to potentiate the post-synaptic actions of GABA, e.g., Etazolate and the other pyrazolopyridine tranquilisers and ethanol. Most interest has been generated by the action of barbiturates, especially those with anaesthetic activity such as pentobarbital. It has been shown by both biochemical and electrophysiological means that in the presence of GABA, barbiturates cause an increase in the mean open time of the Cl^- channel (Study & Barker, 1981). Biochemically this action is manifest as an increase in affinity of benzodiazepine and GABA binding. The action of barbiturates can be blocked by the convulsant, picrotoxinin. The enhancement of GABA binding was only observed when Cl^- or other Eccles ions were included in the medium. The anion specificity for this enhancement parallels the ability of these anions to pass thorough the picrotoxinin-sensitive Cl^- channel. Thus it has been concluded that the barbiturates bind at or near the ion channel of the GABA receptor complex and allosterically modulate the binding of both GABA and benzodiazepines (Olsen, 1981).

2.2.4.4 Convulsant site

Picrotoxin is a nitrogen free substance extracted from the dried berry of the climbing shrub Anamirta cocculus. Picrotoxin consists of two dilactones: picrotin and picrotoxinin. Picrotoxinin is the active component, which is able to block GABAergic synaptic transmission (Olsen et al., 1978b) without blocking GABA binding to its recognition site (Olsen et al., 1978a). Due to picrotoxinin's ability to block the Cl^- channel, tritiated dihydropicrotoxinin was used to study this binding site which must be intimately associated with the ion channel if not the channel itself. However these s[^{3}H]dihydropicrotoxinin the poor signal/noise ratio of [^{3}H]dihydropicrotoxinin binding to brain membranes (Ticku & Olsen, 1978). Further study of this "convulsant" site had to wait for the development of more potent ligands for the site. The labelling of the cage convulsants, synthesised by John Casida (Casida et al., 1976) in the early 1980s has provided very valuable tools for investigating this binding site. This work will be reviewed in the next section.

2.3 The Convulsant Binding Site of the Mammalian GABA receptor-complex

2.3.1 The Connection Between Trioxabicyclooctanes and Picrotoxinin

Trioxabicyclooctanes of the type of structure;

$$Y\text{-}X(OCH_2)_3C\text{-}Z$$

are highly toxic to mice when:

X is phosphorous;

Y is oxygen (phosphate),
sulphur (phosphorothionate),
or not present (phosphite); and

Z is C_2H_5,
$n\text{-}C_3H_7$ or especially,
$i\text{-}C_3H_7$.

On intraperitoneal injection to mice, these compounds cause convulsive seizures and death within a few minutes. The similarity of the manifestations of poisoning to those produced by bicuculline and picrotoxinin, which are GABA antagonists, led to the speculation that these trioxabicyclooctanes were acting in the same way, (Casida et al.,1976). Thus Bowery's group investigated the action of these compounds on single neurons in the rat brain and of the depolarising

action of GABA on the isolated rat superior cervical ganglion. Their results indicated that the convulsant action of the trioxabicyclooctanes may well be related to their ability to antagonise the actions of GABA (Bowery et al., 1976). Additionally the cage convulsants (see below) were found to inhibit [^{3}H]dihydropicrotoxinin binding in rat brain (Ticku & Olsen ,1979).

The trioxabicyclooctanes described above have a conformationally restricted skeleton which resembles a cage. This has given rise to the name "cage convulsants" to describe this general group.

2.3.1.1 Biochemical investigation of the convulsant site

The electrophysiological and biochemical data suggested that the cage convulsants were acting at the same site as picrotoxinin to antagonise the action of GABA. Additionally the very high toxicities of these compounds suggested that they would be much better ligands for the site than [^{3}H]dihydropicrotoxinin. Thus radiolabelled cage convulsants might prove to be very valuable ligands for the biochemical investigation of the convulsant site of the $GABA_A$ receptor complex.

This was first achieved by the groups of Squires and Casida with the synthesis of [^{35}S]t-butylbicyclophosphorothionate (TBPS), (which was easily produced in one step from t-butylbicyclophosphite and elemental ^{35}S) and the characterisation of its binding to rat brain membranes (Squires et al., 1983).

They found that specific [^{35}S]TBPS binding had a temperature optimum near 20 ^{o}C, a pH optimum at pH 8.0 and in the presence of 200 mM KBr was potently inhibited by GABA (IC_{50} = 340 nM). However in 200 mM NaCl, GABA was unable to significantly inhibit the binding. The binding parameters were calculated at pH 7.5, 25 ^{o}C and in 200 mM KBr and were:

K_D = 16 nM and,

B_{max} = 48 pmoles/g of wet brain tissue.

The K_D's and effects of various ligands on the binding of [^{35}S]TBPS in various rat brain regions from a number of workers is shown in table 1. Dissociation of [^{35}S]TBPS was found to be bi- or polyphasic and this was interpreted as indicating the presence of two or more TBPS binding sites.

This work was repeated and extended by Supavilai and Karobath (1984). They found that the halide salt used in the incubation medium had pronounced effects on the binding of [^{35}S]TBPS to rat brain membranes.

Table 1: Comparison of [^{35}S]TBPS binding in rat brain membranes

Source	Brain region	K_D (nM) for TBPS	$E.C._{50}$ & effect of ligand
1	whole in NaCl	50	GABA 90 μM, -'ve Ptx 2 μM, -'ve
2	whole in KBr	25	IBP 0.16 μM, -'ve Ptx 0.4 μM, -,ve GABA 0.45 μM, -'ve Etazolate 5 μM, -'ve Ro5-4864 10 μM, -'ve Pentobarbital 70 μM, -'ve Diazepam > 200 μM, -'ve
3	whole in KBr	16	GABA 340 nM, -'ve Muscimol 59 nM, -'ve Ptx 190 nM, -'ve Pentobarbital 110 μM, -'ve
4	cortex in NaBr in NaCl	 26 60	 Muscimol 23 nM, +'ve 724 nM, -'ve Etazolate 0.4 μM, +'ve 12.9 μM, -'ve Pentobarbital 60.4 μM, +'ve 550 μM, -'ve
5	cortex cerebellum hippocampus in KBr	24	Ptx 0.4 μM, -'ve GABA 0.1-0.8 μM, -'ve Ro5-4864 20 μM, -'ve

1. This thesis
2. Ramanjaneyulu & Ticku (1984)
3. Squires *et al.* (1983)
4. Supavilai & Karobath (1984)
5. Ticku & Ramanjaneyulu (1984)

In the presence of 200 mM NaBr the binding parameters were:

K_D = 26 nM, &

B_{max} = 178 fmole/mg of tissue wet weight.

Whereas if the NaBr was replaced with 200 mM NaCl the binding parameters were found to be:

K_D = 60 nM, &

B_{max} = 193 fmole/mg of tissue wet weight.

Thus the binding sites for TBPS exhibit different affinities with different halide salts.

Like the group of Squires & Casida they found that muscimol was able to inhibit the binding of [^{35}S]TBPS in the presence of NaBr (IC_{50} = 323 nM). However when Supavilai & Karobath replaced the NaBr with NaCl, muscimol gave a biphasic response; a high affinity stimulation (EC_{50} = 23 nM) and a low affinity inhibition (IC_{50} = 724 nM). The EC_{50} and the IC_{50} values for muscimol corresponded to the apparent K_D values of high and low affinity [^{3}H]muscimol binding in the same assay conditions (Supavilai et al., 1982).

Additionally when Supavilai & Karobath studied the effect of benzodiazepines on [^{35}S]TBPS binding in the presence of NaBr, they only observed weak effects of benzodiazepine agonists. However in the presence of NaCl, they found that the benzodiazepine agonists

stimulate the binding of [^{35}S]TBPS, whilst the inverse benzodiazepine agonists inhibited the binding.

Like the effects of muscimol Supavilai & Karobath were able to reproduce the inhibition of [^{35}S]TBPS binding by etazolate and pentobarbital (IC_{50}s = 2.8 x 10^{-6} M and 260 x 10^{-6} M, respectively) reported by Squires & Casida. However in the presence of NaCl etazolate and pentobarbital exhibited a biphasic action on [^{35}S]TBPS binding, with stimulation at lower concentrations (EC_{50}s = 0.4 x 10^{-6} M and 60.4 x 10^{-6} M, respectively) and inhibition at higher concentrations (IC_{50}s = 12.9 x 10^{-6} M and 550 x 10^{-6} M, respectively). Supavilai & Karobath further observed that the Hill number for etazolate inhibition was 1.5 and that for pentobarbital inhibition was 2.7. Supavilai & Karobath concluded that the biphasic actions of etazolate and pentobarbital, and the Hill numbers > 1 for their inhibitory effects indicated a mulitiplicity of TBPS binding sites in the GABA benzodiazepine receptor complex, with cooperativity between these sites. This was also suggested by the biphasic dissociation reported by Squires et al. (1983).

Further evidence of multiple TBPS sites was presented by Ticku & Ramanjaneyulu (1984) who examined the effect of the atypical benzodiazepine, Ro5-4864. Ro5-4864 is a 1,4-benzodiazepine which differs from diazepam by a chlorine substituent at the para position

of the benzene ring and unlike other benzodiazepines has been shown to produce convulsions. Ticku & Ramanjaneyulu found that Ro5-4864 was able to inhibit [^{35}S]TBPS binding in a dose-dependent manner (IC_{50} = 20 x 10^{-6} M). The Hill number for the displacement curves was significantly less than 1. Ro5-4864 decreased the affinity of [^{35}S]TBPS binding, without altering the B_{max}, which suggests a competitive type of inhibition. They also found that picrotoxinin did not inhibit the high-affinity [^{3}H]Ro5-4864 binding to rat brain membranes. Ticku & Ramanjaneyulu concluded that the Hill coefficients less than 1 for the displacement of TBPS binding by Ro5-4864 suggest that Ro5-4864 may distinguish between subtypes of TBPS binding sites.

In summary a number of groups have been able to show high-affinity binding of [^{35}S]TBPS to brain membranes, which is affected by halide ions, allosterically modified by GABA agonists, benzodiazepine agonists, benzodiazepine inverse agonists and tranquilisers such as etazolate and barbiturates.

2.3.1.2 Insecticides and the convulsant binding site

Cyclodiene insecticides were known to induce strong nerve excitation, however by 1973 the site of action of these insecticides had only been pin-pointed to the synaptic region (Shankland & Schroeder, 1973). Although by chemical definition lindane is not a cyclodiene, it appears to have a very similar mode of action. This is most clearly seen with resistant species. Cyclodiene resistant insects are almost always resistant to lindane and vice versa (Grayson, 1954; Butts & Davidson, 1955). In 1982 Ghiasuddin & Matsumura developed a bioassay system using the coaxal muscle of the American cockroach to measure the stimulatory effect of GABA on $^{36}Cl^-$ permeability which was antagonised by picrotoxinin. Using this system they were able to show that heptachlor epoxide (a cyclodiene) and lindane antagonised the stimulatory effect of GABA (Ghiasuddin & Matsumura, 1982). Additionally they found that these two cyclodiene-type insecticides were competitive inhibitors of [^{3}H]dihydropicrotoxinin binding and that several cyclodiene resistant insect strains were also resistant to picrotoxinin, but not other neuroexcitants such as bicuculline (Matsumura & Ghiasuddin, 1983). This led to the speculation that cyclodiene insecticides acted

through the convulsant binding site of the GABA receptor complex.

The later pyrethroid insecticides, esters of (S)-alpha-cyano-3-phenoxybenzyl alcohol with various 3-substituted-2,2-dimethylcyclopropane-carboxylic acids (such as cypermethrin and deltamethrin) or 2-substituted-3-methylbutyric acids (such as fenvalerate) are potent and widely used insecticides. These later pyrethroids have a poisoning syndrome distinct from that of early pyrethroids, which is designated type 2. Type 2 syndromes closely resemble the action of picrotoxinin in mammals, and it was subsequently found by Lawrence & Casida (1983) that such type 2 pyrethroids were competitive inhibitors of [^{35}S]TBPS binding in rat brain membranes, indicating that the type 2 pyrethroids may also act through the convulsant site of the GABA receptor complex. However cypermethrin inhibition of TBPS binding was mixed or possibly non-competitive. This suggests that the pyrethroid binding domain may be distinct from that of TBPS or that they only partially overlap (Lawrence & Casida, 1983).

In 1984 Lawrence & Casida then demonstrated that the cyclodiene type insecticides (including lindane) were also potent, competitive inhibitors of TBPS binding in rat brain membranes.

Thus there was evidence that the cyclodiene type and type 2 pyrethroid insecticides may be acting through the convulsant site of the GABA receptor complex. However the biochemical investigation of these interactions using the potent ligand, TBPS was done in rat brain. Our laboratory had previously shown, with studies on the GABA and benzodiazepine binding site that there were subtle differences between the locust and mammalian GABA receptors. What was not known was whether the locust had a convulsant binding site and whether it was the site of action of these insecticides. Thus this study was initiated to investigate the convulsant binding site of the Desert Locust using the potent cage convulsant TBPS.

2.4 Avermectins & GABA

Avermectins are a family of macrocyclic lactones isolated from Streptomyces avermitilis (Burg et al., 1979) which have potent anthelmintic and insecticidal activity (Egerton et al., 1979). In 1982 it was proposed that these compounds act by interferring with the GABA receptor in neuronal tissue (Pong & Wang, 1982). Calcott & Raymond (1984) found that avermectin stimulated [^{3}H]GABA binding in rat brain and brine shrimp membranes whereas it was a competitive inhibitor of GABA binding in the fungus, Mucor miehei. Tanaka & Matsumura (1985) found that on the leg muscles and nervous system of the

American cockroach, avermectin B1a was able to open chloride channels. However its action was not directly through the GABA, benzodiazepine or convulsant binding sites. Olsen & Snowman (1985) found that in rat brain membranes avermectin B1a could enhance the binding of GABA and diazepam. Then in 1986 Abalis & Eldefrawi (Abalis et al., 1986) found that [^{3}H]muscimol binding to honeybee brain membranes was potently inhibited by avermectin B1a. This is not a thorough review of the work on avermectins but it does serve to illustrate how difficult it has been to define a mode of action for these compounds. However more recently workers have come to a consensus that the avermectins most potent action is to open chloride channels which are not part of a GABA receptor complex (Nicholson et al.,1988 & Schaeffer et al, 1988). However it is evident from the small selection of work above that avermectins may have some kind of effect on the GABA receptor complex chloride channels.

2.5 The Invertebrate GABA System

The invertebrate GABAergic system has been very thoroughly reviewed by Robinson & Olsen (1988) and I will only provide a brief summary of the similarities and differences between invertebrate and vertebrate GABA transmission.

2.5.1 GABA Synthesis & Metabolism

As mentioned previously it was the work of Kravitz in the 1960's on the invertebrate, (lobster), that led to the detailed study of the GABA system in vertebrates. However since this early work, whilst GAD and GABA-T have been purified from various vertebrate tissues, there has been very little work on invertebrate GABA-T and only slightly more on GAD. Whilst differences were found between unpurified invertebrate enzymes and their vertebrate counterparts, GAD (Stapleton 1986) and GABA-T (Jeffery et al. 1988) have recently been purified from insect tissues and found to be essentially the same as the mammalian enzymes.

2.5.2 GABA Uptake

Some of the early demonstrations of GABA uptake were in invertebrate preparations, (Iversen, & Kravitz, 1966) in whole abdominal muscles of the lobster. This work stimulated the study of GABA uptake in mammalian synaptosomes. However no method existed for the

production of invertebrate synaptosomes and studies had to concentrate on tissue slices, whole nerves and muscles. It was not until 1980 that a method for producing insect synaptosomes was published by Breer & Jeserich, which allowed Gordon et al. (1982) to produce synaptosomes from locust nervous tissue which possessed an active GABA transport mechanism. Additionally Robinson & Lunt (1986) have used a rapid method for the production of a preparation with a high density of synaptosomes, from locust CNS, which posses a GABA uptake system. Thus evidence is appearing that insect CNS possesses GABA uptake systems as well as those initially described in invertebrate neuromuscular preparations. Such systems may well play a part in the removal of GABA from the synapse.

2.5.3 A GABA Receptor Complex

2.5.3.1 GABA binding site

The first invertebrate GABAergic binding studies were performed in crayfish muscle by Meiners et al. (1979) who demonstrated specific, saturable, Na^+-independent binding of [^{3}H]muscimol. However it was only recently that workers have become interested in invertebrate CNS GABA binding sites. Several groups have probed insect CNS with [^{3}H]muscimol and [^{3}H]GABA (Lummis & Sattelle, 1985a; Lunt et al., 1985; Tanaka & Matsumura, 1985; Abalis & Eldefrawi, 1986; Breer &

Heilgenberg, 1986). A common finding in all these studies is a lack of any sensitivity of insect CNS GABA binding sites to the mammalian $GABA_A$ antagonist, bicuculline, whilst they are sensitive to $GABA_A$ agonists. The details of these invertebrate binding studies are compared with the data from a selection of mammalian GABAergic studies (see table 2). The lack of bicuculline sensitivity in invertebrate GABAergic binding studies is in agreement with electrophysiological studies by Beadle et al. (1985) on cultured locust neurones.

2.5.3.2 Benzodiazepine binding site

Nielsen et al. (1978) reported that benzodiazepine binding sites were present in a wide range of vertebrate species but absent in the nervous system of five invertebrate species (earthworm, squid, woodlouse, lobster and locust). They thus suggested that brain-specific benzodiazepine receptors had a late evolutionary appearance. Subsequently, a number of workers have demonstrated the existence of benzodiazepine receptors in various insect preparations. Abalis et al. (1983) demonstrated the presence of [^{3}H]flunitrazepam binding sites in a house fly thoracic preparation. Tanaka & Matsumura (1985) observed binding of [^{3}H]diazepam in cockroach ganglia. Using a filtration assay, Robinson et al. (1986) have characterised a

Reference	1	2	3	4	5	6	7	8	9	10
K_D(nM)	10	40	30	3 and 144	384	100	9	5 and 30	370	100
B_{max} (fmol/mg protein)	70	20	200	50 and 500	1420	2200	500	500 and 900	680	—
Ligand [^{3}H]	Muscimol	Muscimol	GABA	Muscimol	GABA	GABA	Muscimol	Muscimol	GABA	GABA
Tissue	Locust ganglia	Housefly head	Locust ganglia	Honeybee brain	Cockroach CNS	Locust ganglia	Crayfish muscle	Rat brain	Rat brain	Rat brain
IC_{50}(M) muscimol	3×10^{-8}	3×10^{-8}	1×10^{-7}	6×10^{-9}	7×10^{-7}	9×10^{-4}	2×10^{-8}	1×10^{-8}	—	4×10^{-8}
GABA	1×10^{-7}	4×10^{-8}	9×10^{-8}	4×10^{-8}	1×10^{-7}	—	2×10^{-7}	4×10^{-8}	4×10^{-7}	2×10^{-7}
Isoguvacine	2×10^{-7}	1×10^{-7}	5×10^{-5}	—	5×10^{-4}	—	6×10^{-8}	—	—	2×10^{-7}
3-APS	3×10^{-6}	3×10^{-7}	—	3×10^{-5}	4×10^{-4}	NS	3×10^{-6}	3×10^{-8}	3×10^{-7}	6×10^{-7}
Imidazole acetate	—	6×10^{-7}	—	1×10^{-6}	—	—	2×10^{-7}	2×10^{-7}	2×10^{-7}	9×10^{-7}
Bicuculline	$>10^{-3}$	4×10^{-4}	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	NS	3×10^{-3}	6×10^{-6}	4×10^{-6}	4×10^{-6}
Chlorpromazine	—	—	—	—	—	1×10^{-7}	—	—	2×10^{-4}	—
Imipramine	—	—	—	—	—	1×10^{-7}	—	—	—	—
2,4-DABA	—	$>10^{-3}$	—	$> 10^{-3}$	—	NS	$>10^{-3}$	$>10^{-5}$	$>10^{-3}$	$>10^{-4}$
Nipecotic acid	—	$>10^{-4}$	$>10^{-3}$	$>10^{-4}$	$>10^{-3}$	—	$>10^{-3}$	$>10^{-5}$	—	$>10^{-4}$
β-Alanine	—	—	$>10^{-3}$	5×10^{-6}	$>10^{-3}$	—	—	$>10^{-5}$	8×10^{-5}	4×10^{-5}

Sources: 1, Robinson (1986). 2, Lunt *et al.* (1985). 3, D. Jeffery, (personal communication). 4, Abalis and Eldefrawi (1986). 5, Lummis and Sattelle (1985a). 6, Breer and Heilgenberg (1986); 'NS' denotes 10% displacement of binding, maximum concentration of ligand not given. 7, Meiners *et al.* (1979). 8, Williams and Risley (1979). 9, Enna and Snyder (1975). 10, Greenlee *et al.* (1978b).

Table 2. Comparison of GABAergic binding studies in invertebrate and vertebrate nervous tissues

heterogenous population of benzodiazepine receptors using [^{3}H]flunitrazepam in a locust CNS preparation. Also using [^{3}H]flunitrazepam, Lummis & Sattelle (1985b) have shown benzodiazepine binding sites in cockroach CNS. A common feature of these studies was that the peripheral benzodiazepine, Ro5-4864 was more potent an inhibitor of [^{3}H]flunitrazepam binding, than the central benzodiazepine, clonazepam (except for the study of Tanaka & Matsumura, who did not study the effect of other benzodiazepines). Despite the peripheral pharmacology suggested by these results these insect CNS benzodiazepine receptors were affected by GABA and Robinson et al. (1986) were able to photoaffinity label the locust CNS binding site with [^{3}H]flunitrazepam. Both these properties are characteristic of the central benzodiazepine binding site (Mohler et al. 1980), in mammals and not the peripheral binding site (Thomas & Tallman, 1981). Additionally Robinson et al. (1986) reported that it was necessary to replace the physiological concentrations of calcium which were washed out during the membrane preparation to obtain [^{3}H]flunitrazepam binding. They interpreted this as indicating a difference in cation requirements from the mammalian central benzodiazepine binding site.

2.5.3.3 Convulsant binding site

Tanaka et al. (1984) reported the specific binding of picrotoxin to a preparation from cockroach CNS. This was further evidence that insect CNS contained a GABA receptor complex very similar to the $GABA_A$ receptor complex in mammals.

This thesis will describe work done in our laboratory to characterise the convulsant site in locust CNS using the cage convulsant TBPS. Work by other groups on TBPS binding in insect tissues will be discussed in chapter 6.

Chapter 3 : Materials

Locusts, (Schistocerca gregaria), in the 5th instar were supplied by Larujohn, Colwyn Bay, U.K. and Cambridge Bioscience Services, Chesterton Hall Crescent, Cambridge, U.K.

250g Wistar Rats were obtained from the University's Animal House.

General reagents were obtained from B.D.H Chemicals, Poole, Dorset, U.K. unless otherwise stated. Picrotoxinin was obtained from Sigma Chemical Company Ltd., London, U.K.

Dieldrin was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Isopropylbicyclophosphate and lyophilised Picrotoxinin were gifts from Dr. R.W. Olsen, UCLA, California, U.S.A.

Cypermethrin, Bendiocarb, Carbofuran, Dimethoate and Lindane were gifts from Dow Chemical Company, Letcombe Manor, nr. Wantage, U.K.

Unlabelled benzodiazepines were a gift from Hoffmann-La Roche Ltd.

The GABA agonists, isoguvacine-HCl and 3-aminopropane sulphonate and the antagonist, bicuculline methiodide were obtained from Cambridge Research Biochemicals Ltd. Cambridge, U.K.

[^{35}S]TBPS (specific activity of 70 Ci.mmole) and TBPS were obtained from New England Nuclear, Boston, U.S.A.

Na^{36}Cl (specific activity of 66 Ci/mmole) was obtained from Amersham International plc, Amersham, Buckinghamshire, U.K.

Radioactivity was determined by liquid scintillation spectrometry using a Packard Minaxia Tri-carb 4000 model counter and Optiphase scintillation fluid, supplied by LKB, Loughborough, Leistershire, U.K. The efficiency for ^{35}sulphur was 70% and for ^{36}chlorine was 95%.

Soluene tissue solubiliser was obtained from Packard Instrument Company.

Centrifugation procedures were carried out using a Dupont Instruments Sorvall RC-5B, a Beckman L5 50B ultracentrifuge and a MSE micro centaur.

Spectrophotometric assays were performed using a Pye Unicam SP6-450.

Chapter 4 : Methods

4.1 Preparation of the P2 Membrane Fraction from the supraoesphogeal ganglia of the Locust.

Locusts were first stunned by exposure to carbon dioxide (CO_2), usually in the form of 'dry-ice'. They were then decapitated and their heads collected in an ice cold container. The supraoesophageal ganglion was exposed by cutting away the cuticle, from the mouthparts, through the compound eye, to the top of the head. (fig. 6) The ganglia were then removed onto ice cold aluminium foil with small, watch repairer's forceps. The required number of ganglia were then homogenised:

35 passes in a 30 ml glass/teflon, motor driven, Potter-Elvejham (radial clearance 0.15 mm) homogeniser, setting 2.5 on a Citenco motor, with 5 ml of 10 mM tris[hydroxymethyl]-aminomethane,(Tris) buffer, containing; 0.25 M sucrose and 1 mM ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetra acetic acid(EGTA) at pH 7.4 (buffer-S). This was followed by 5 passes in a 10 ml glass/glass homogeniser, supplied by Uniform, Jencons, U.K. The homogenate was then decanted into an ice cold, Sorvall SS34 centrifuge tube. The homogenisers were washed out with ice cold buffer-S and the washes added to the homogenate to a volume of around 35 ml and was

Figure 6: View of the top of the head of the locust with the cuticle shaved away expose the supraoesophageal ganglion, which is shown dissected out below. Scale is in mm.

then centrifuged at 500 x g(av.) for 5 min. The supernatant was filtered through nylon bolting cloth (159 um mesh size) into an ice cold Beckman S35 centrifuge tube. The pellet was resuspended in a further 35 ml of ice cold buffer-S and the above procedure repeated. The second supernatant was combined with the first in the S35 tube.

The combined supernatants were then centrifuged at 150 000 x g(av.) for 30 min. The resultant supernatant was discarded and the pellet was resuspended in approximately 70 ml of ice cold buffer-S and left to stand on ice for 30 min. After which time the above high speed spin was repeated to yield the final pellet P2, which was resuspended in 10 mM NaH_2PO_4 buffer containing 200 mM NaCl at pH 7.4, (buffer-A) to give the required membrane protein concentration. Finally the membrane preparation was again homogenised in the 10 ml glass/glass homogeniser, 10 passes.

4.2 Assay of [^{35}S]TBPS Binding to Locust Ganglia P2 Membranes

Membranes (P2) were suspended (2-5 mg of membrane protein / ml) in buffer-A.

Total binding was obtained by incubating 80 ul of membrane preparation at room temperature (22-24 ^{o}C) for

three hours in the presence of 10 µl of [^{35}S]TBPS (3-600 nM, final concentration) and 10 µl of buffer-A .

Non-specific binding was defined as that obtained by incubating 80 µl of membrane preparation as above, but replacing 10 µl of buffer-A with 10 µl of buffer-A containing IBP(0.1 mM) , or TBPS(4.5 µM) (final concentrations).

Bound [^{35}S]TBPS was separated from free [^{35}S]TBPS by filtration over vacuum on a Millipore filtration manifold (12 filter size) using Whatman GF/B filters (2.5 cm diameter), after pre-dilution of the incubation mixture with 3.5 ml of ice cold buffer-A. The incubation tubes were then washed twice with 3.5 ml of ice cold buffer-A and the washes filtered on the same GF/B filter. The filters were then placed in scintillation vials with 5 ml of Optiphase scintillation fluid. The vials were then counted in a Packard Minaxia liquid scintillation counter (regions 0-1700).

Each concentration of [^{35}S]TBPS was assayed in triplicate for both total and non-specific binding.

Specific binding was calculated by subtraction of non-specific binding from total binding.

The total number of binding sites (B_{max}) and dissociation constant (K_D), for [^{35}S]TBPS binding were estimated by:

Scatchard analysis (Scatchard, 1949); Hill analysis (Hill, 1910); and computer programmes for a) direct linear plot (Enzpac, Elsevier-Biosoft(TM), Cambridge, U.K.) and b) 'By Hand' curve-fitting program (Humrich & Richardson 1983).

4.3 Measurement of the Dissociation Rate of [^{35}S]TBPS Binding to Locust Ganglia P2 Membranes

4.3.1 Infinite Dilution

Membranes were suspended in buffer-A (1-3 mg membrane protein / ml).

Membrane preparation (0.9 ml) was incubated with 0.1 ml of 100 nM [^{35}S]TBPS (final concentration of 10 nM in the assay) at room temperature for three hours, to allow the system to reach equilibrium.

After this time 99 ml of buffer-A were added to the incubation mixture and mixed continually with a magnetic stirrer. Samples (10 ml) were removed at time intervals, zero (just after time of addition of 99 ml of buffer) and onwards.

Each sample was immediately filtered over vacuum on a Millipore filter manifold using Whatman GF/B filters.

The filters were then washed and the radioactivity counted as described in 4.2.

4.3.2 Dilution of Radiolabel

The same incubation was carried out as above in 4.3.1.

After the system has reached equilibrium (3 hours), 4 µl of unlabelled TBPS (45 mM) were added to the incubation and mixed thoroughly.

Samples (100 µl) were removed at time, zero and onwards.

The samples were added to tubes containing 3.5 ml of ice cold buffer-A, mixed and filtered over vacuum as in 4.3.1. The filters were then washed and the radioactivity counted as described in 4.2.

For both methods the dissociation rate constant was estimated by plotting:

$$\ln (B) / (B_0) \text{ versus time}$$

4.4 Measurement of the Association Rate of [^{35}S]TBPS Binding to Locust Ganglia Membranes

Membranes were suspended in buffer-A (1-3 mg of membrane protein/ml).

One tube was set up, as below, for each time point between time zero and 180 min.

Each tube contained 90 ul of membrane preparation and 10 ul (10-40 nM final concentration) of [^{35}S]TBPS.

The reaction was started by the addition of the [^{35}S]TBPS and stopped at the correct time point by the addition of 3.5 ml of ice cold buffer-A.

The contents of each tube were then rapidly filtered over vacuum as previously described in 4.2.

The tubes and filters were then washed and the radioactivity on the filters counted as described in 4.2.

The data at each concentration of [^{35}S]TBPS were plotted as:

$$\log (B_{eqm}) / (B_{eqm} - B_t) \text{ against time } (t)$$

The slope of each line was then plotted against concentration of [^{35}S]TBPS.

The slope of this secondary plot is the association rate constant and the intercept with the ordinate is the dissociation rate constant.

4.5 Competition Binding Studies

To measure the potency of other ligands at displacing TBPS from the binding site in locust ganglia P2 membranes the following method was used.

Membrane preparation (80 µl) was incubated at room temperature for 3 hours with;

10 µl [^{35}S]TBPS at 10-20 nM (final concentrations) and 10 µl of either:

buffer-A, IBP or TBPS, or various concentrations of the ligand being tested.

For control purposes the buffer-A contained the same concentration of ethanol and any other solvent introduced with the IBP, TBPS or other test ligands.

4.6 Preparation of Rat Brain P2 membranes

Frozen rat brain (minus the hippocampus) was homogenised (10% w/v) in 0.32 M sucrose containing, 1 mM ethylenediaminetetracetic acid (EDTA), 0.1 mM phenylmethylsulphonylfluoride (PMSF) and 0.1% NaN_3 at pH 7.4.

The homogenate was then centrifuged in SS34 tubes in a Sorvall R5CB at 1000 x g(av.) for 10 min.

The supernatant was decanted off and left on ice, whilst the pellet was resuspended in approximately half the original volume of sucrose and re-centrifuged.

The two supernatants were combined and centrifuged at 17000 x g(av.) for 20 min.

The pellet was resuspended in 50 mM phosphate buffer at pH 7.4 containing: EDTA, PMSF and NaN_3 (at the same concentration as they were present in the sucrose).

The suspension was then centrifuged at 17000 x g(av.) for a second time.

The pellet was again resuspended in phosphate buffer and the centrifugation repeated a third time.

The final pellet was resuspended in TBPS assay buffer (buffer-A) at approximately 2 ml/g of original wet weight and then diluted as required.

4.7 Assay of [^{35}S]TBPS binding to Rat Brain membranes

Binding of [^{35}S]TBPS to rat brain membranes was carried out exactly as for the locust ganglia membranes.

Competition binding experiments were also the same.

4.8 Preparation of Locust ganglia microsacs:

4.8.1by washing the homogenate using centrifugation, (after the method of Wafford et al. (1987)).

The required number of ganglia were dissected out and then homogenised (100 ganglia in 20 ml) in 10 mM NaH_2PO_4 containing: 5 mM KCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM glucose, pH 7.5 with Tris base (buffer-F) using a motor

driven, 30 ml glass/teflon homogeniser with the motor set at its lowest speed.

The homogenate was then dispersed into 1 ml eppendorf tubes and centrifuged at high speed (11500 x g) for 10 min in a bench microfuge. The supernatants were discarded and the pellets resuspended in 1 ml of buffer-F. The centrifugation step was then repeated. The final pellets were resuspended in 250 ul of buffer-F and pooled.

4.8.2 without washing by centrifugation,

(after the method of Robinson (1986)).

The required number of ganglia were dissected out as normal but homogenised very gently in a 1 ml glass/teflon homogeniser by hand, making 5 passes extremely slowly, on ice. The ganglia were homogenised in groups of 15 in 1 ml of buffer-F and then pooled. The pooled homogenate was then filtered through nylon boulting cloth of 159 um pore size and diluted as necessary for the assay.

4.9 Assay of ^{36}Chloride Flux into microsacs prepared from Locust ganglia

Microsac preparations (200 µl) were incubated for 10 min. at 30 °C. $Na^{36}Cl$, (200 µl, specific activity of 1.2 x 10^{-3} Ci/mmole) ± 10^{-4} M GABA were added to initiate the assay. The assay was stopped by the addition of 4 ml of ice cold buffer-F and then rapidly filtered over vacuum using a single tower Millipore filtration unit and Whatman GF/C filters. Test ligands were added with the radiolabelled chloride. Optiphase scintillation fluid was added to the filters in vials and the radioactivity counted using a Packard Minaxia liquid scintillation counter.

4.10 Assay of ^{36}Chloride Flux in Whole ganglia from the Locust

Ganglia (one per assay tube) were very carefully removed and then cut twice at right angles. The cut ganglia were then placed in 250 µl of aerated Locust Ringers saline (10 mM KCl, 140 mM NaCl, 6 mM NaH_2PO_4, 4 mM $NaCO_3$, 4 mM $CaCl_2.2H_2O$, 90 mM sucrose, pH 6.8) and left to equilibrate for 45-60 min. Test ligands, if any, were added during the last 30 min. The assay was initiated with 250 µl $NaCl^{36}$ (specific activity of 1.2×10^{-3} Ci/mmole) $\pm$ 5×10^{-4} M isoguvacine.

The assay was stopped with 3 ml of ice cold Locust Ringers saline and then filtered over vacuum as in 4.9. Each tube was washed out with 2 x 3 ml of ice cold Locust Ringers saline. Soluene-350m (0.5 ml) was added to each filter in a scintillation vial and then left overnight at room temperature to solubilise the ganglion. Glacial acetic acid (0.5 ml) was then added to neutralise the soluene followed by 5 ml of Optiphase liquid scintillation fluid. The radioactivity in each vial was then counted as in 4.9.

4.11 Protein Estimation

Protein estimation was by the method of Lowry *et al.* (1951), except reagent A always contained sodium dodecyl sulphate (5% w/v) to enable protein in buffers containing sucrose, EDTA & EGTA to be assayed. Bovine serum albumin was the standard protein.

Chapter 5 : Results

5.1 [^{35}S]TBPS Binding to Locust Ganglia P2 membranes

For a typical Scatchard analysis, (as described later) at least 50 locusts would be required.

The non-specific binding was defined as that in the presence of 0.1 mM IBP or 4.5 μM TBPS. The specific binding of [^{35}S]TBPS was 33% ± 3.3% (n=9) when non-specific binding was defined using IBP and 67% ±5% (n=11), when using TBPS, of the total binding to locust ganglia membranes.

5.1.1 Linearity of [^{35}S]TBPS binding with variation of P2 membrane concentration

The change in [^{35}S]TBPS binding with increasing membrane concentration was measured so the limit of the linear response could be found and the experimental membrane concentration kept below this. The binding was linear over the range 0.024 - 5.0 mg of membrane protein / ml, reaching a plateau at 7 mg of membrane protein / ml, see fig. 7. Most experiments were carried out in the range 1-5 mg of membrane protein / ml.

5.1.2 pH dependence of [^{35}S]TBPS binding to Locust ganglia membranes

The pH dependence of the binding protein for [^{35}S]TBPS was studied over the range pH 5.0 - 9.5. 10 mM Tris-acetate pH 5.0, 10 mM NaH_2PO_4 pH 6.4 - 7.5, and 10 mM Tris-HCl pH 8.0 - 9.5, and Na-

glycinate pH 10.5 - 11.0, were the buffers used to construct the profile. All buffers contained 200 mM NaCl.

The profile in fig. 8 shows a marked optimum at pH 9.0.

All other experiments were carried out at pH 7.4, which gave approximately 50% of the optimum binding. This was also the pH used in previous studies on mammalian brain (Squires et al., 1983) and the pH most commonly used to demonstrate the modulation of 'GABAergic' binding sites by GABAergic ligands (Olsen 1981). Thus if this study was to detect modulation of the cage convulsant binding site by GABAergic ligands it would be advantageous not to be at maximum binding (so any change can be dtected) and to avoid extremes of pH which may not be optimal for these other binding sites.

5.1.3 Chloride dependence of [^{35}S]TBPS binding to Locust ganglia membranes

The chloride dependence of the binding was studied over the range 25 - 350 mM NaCl and found to be linear over those concentrations (fig. 9). The line did not extrapolate back to the origin indicating that the binding was enhanced by Cl^-, but that there was no absolute requirement for it.

5.1.4 Scatchard analysis of [^{35}S]TBPS binding to Locust ganglia membranes

Each scatchard analysis was carried out over a range of at least eight concentrations of [^{35}S]TBPS. Initially a concentration range of 15 - 150 nM [^{35}S]TBPS was used, as such a concentration range would cover and exceed 3 fold, the reported K_D for mammalian

brain (Squires et al., 1983). The binding isotherm for 3 such experiments is shown in fig. 10a. The binding appears to saturate at around 50 nM but then increases again and is not saturated at 150 nM [^{35}S]TBPS. Scatchard analysis of these data gave a line with a very shallow gradient, as the binding had not reached saturation.

The apparent "high-affinity" site was further investigated by using a [^{35}S]TBPS concentration range of 2 - 30 nM. However these concentration ranges gave binding isotherms which were sigmoidal, (see fig. 10b).

Additionally when the data from 3 experiments in the 2 - 30 nM range were combined with data from 3 experiments in 15 - 150 nM range on a Scatchard plot a curvelinear line was obtained which was concave downwards, (see fig. 11).

A Hill analysis of these combined data is shown in fig. 12 and also shows deviation from the linear. Extrapolation of the line after the deviation gives an estimate of the K_D for TBPS of 31.5 nM.

Due to the cost of [^{35}S]TBPS and the short half-life of ^{35}S the characterisation of TBPS binding to locust ganglia membranes at higher concentrations, up to 600 nM, was only done 3 times.

The binding over these higher concentrations appears to saturate at about 500 nM, (see fig. 14). Using a least squares dose / response curve fitting program, estimates of the binding parameters were obtained.

$F_{0.5}$ = 417 ± 74 nM

B_{max} = 1083 ± 256 fmoles / mg of membrane protein.

± S.E.M, n = 3. $F_{0.5}$ = the concentration required to give 50% occupancy of the binding sites (see 6.1.3).

5.1.5 Measurement of the association rate of [^{35}S]TBPS binding to Locust ganglia membranes

5.1.5.1 Calculation of the association rate constant

Association experiments were carried out at 10, 20 ,30, and 40 nM [^{35}S]TBPS.

When plots of :

$$\ln (B_{eqm}) / (B_{eqm} - B_t) \text{ vs. time}$$

were drawn for each concentration, they were clearly not linear, (see figs. 15 & 16). There seemed to be two components to the association, an early stage, up to 30 min and a later stage from 60 - 180 min. The non-linear nature of this data indicate that the binding was not demonstrating Michaelis-Menten kinetics and thus derivation of the rate constant for association would be invalid. However approximate gradients were obtained from each component to

give an observed association rate constant (k_{1obs} see table 3), so that the secondary plot:

k_{1obs} vs. concentration of [^{35}S]TBPS

could be plotted, (see fig. 17). To further emphasise the non-linear nature of the data; both components of the association changing with increasing concentration of [^{35}S]TBPS. The early component appears to increase, as expected, with increasing TBPS concentration. However after reaching a maximum at 20 nM [^{35}S]TBPS it decreases, reaching its starting value at 40 nM TBPS. The later component, however, seems independent of TBPS concentration until 30 nM and then rapidly increases. Approximate gradients were obtained for the early and later components which are equal to k_1, the association rate constant.

k_1, early component = 0.23 fmoles/mg of membrane protein^{-1} min^{-1},

later component = 0.26 fmoles/mg of membrane protein^{-1} min^{-1}.

These data indicate that the association rate constant k_1 was changing at different concentrations of TBPS and thus they are not 'true constants'.

5.1.5.2 Measurement of the association rate of [^{35}S]TBPS binding to Locust ganglia membranes in the presence of TBPS

When association experiments were done using locust ganglia membranes that had been pre-incubated with small concentrations of unlabelled TBPS, an anomalous enhancement of [^{35}S]TBPS binding was observed, (see fig. 18).

5.1.6 Measurement of the dissociation rate for [^{35}S]TBPS binding to locust ganglia membranes

Figure 19 shows a plot of:

$\%B_0$ vs. time

for both methods of determining the dissociation rate constant described in 4.3. A linear relationship was obtained when infinite dilution was used. However when dilution of the radiolabel was used to initiate dissociation a curvelinear response was obtained, indicating that k_{-1} was also changing with concentration of TBPS and was thus also not a 'true constant'. The significance of the non-linear association and dissociation will be covered in Chapter 7.

Initial dissociation rate constants were estimated from plots of:

$$[B_t]/[B_0] \text{ vs. time}$$

(see figs 20 & 21).

Infinite dilution gave a dissociation rate constant (k_{-1}) of:

$$9.5 \times 10^{-3} \text{ min}^{-1}.$$

Dilution of the bound radiolabel with an excess of unlabelled TBPS gave a dissociation rate constant of:

$$28.6 \times 10^{-3} \text{ min}^{-1}.$$

After 30 min the radiolabel starts to reassociate, reaching a stable equilibrium at 50 min (see fig. 21). This equilibrium was still steady after 180 min (not shown in fig. 21).

Later Scatchard analysis

Twice, restriction of the [^{35}S]TBPS concentration range between 10 & 50 nM, resulted in linear Scatchard plots which give estimates for the binding parameters of:

K_D = 34 nM

B_{max} = 288 fmoles / mg of membrane protein.

See fig. 13. However these experiments were carried out at 5-fold higher membrane concentrations, the significance of this observation

See fig. 13. However these experiments were carried out at 5-fold higher membrane concentrations, the significance of this observation will be covered in Chapter 7.

5.1.7 Competition by other ligands for the TBPS binding site in Locust ganglia membranes

Table 4 shows a summary of the effects and potencies of the ligands tested against [^{35}S]TBPS binding in locust ganglia membranes. E.C.$_{50}$ is the concentration of a ligand which is required to give 50% of the maximum effect of that ligand, either inhibitory or facilitatory. Picrotoxinin, was ineffective at displacing TBPS from its binding site in locust ganglia membranes, even at 10^{-4} M.

The only ligands tested which were found to inhibit [^{35}S]TBPS binding to locust ganglia membranes were the cage convulsants, TBPS itself and isopropylbicyclophosphate(IBP). The inhibition curves are shown in fig. 22, TBPS was approximately 3 fold more potent than IBP. The effects of GABA and Ro5-4864 on the inhibition by TBPS over a limited concentration range were also studied:

	IC_{50}	Hill no.	correlation coefficient
control	20 nM	1.4	1.0
+GABA	22 nM	1.0	0.97
+Ro5-4864	0.8 nM	1.5	0.99

GABA (fig. 23): the benzodiazepines, clonazepam Ro5-4864 (fig. 24) & diazepam and pentobarbital (fig. 25) all enhanced the binding of [^{35}S]TBPS binding to locust ganglia membranes. Ro5-4864 was more effective at enhancing [^{35}S]TBPS binding, than clonazepam (larger maximum binding), but clonazepam was 2 fold more potent than Ro5-4864 (smaller EC_{50}). Pentobarbital was the most potent enhancer.

Although picrotoxinin was ineffective in locust ganglia membranes, some insecticides were potent enhancers of [^{35}S]TBPS binding (fig. 26). The Type I pyrethroid, permethrin had no effect at 0.1 mM.

5.2 Confirmation of Previous Data for [^{35}S]TBPS Binding to Rat Brain Membranes

5.2.1 Calculation of the binding parameters K_D & B_{max} for [^{35}S]TBPS binding to rat brain membranes

The binding of [^{35}S]TBPS to rat brain membranes was carried out over the range 1 - 120 nM [^{35}S]TBPS. The binding parameters were estimated using a computer program for least squares dose / response curve fitting.

$K_D = 53 \pm 2.7$ nM

$B_{max} = 306 \pm 63.3$ fmoles / mg of membrane protein.

$\pm$ S.E.M, n = 3.

5.2.2 Effect of Picrotoxinin & GABA on [^{35}S]TBPS binding to rat brain membranes

Both picrotoxinin and GABA were able to inhibit [^{35}S]TBPS binding to rat brain membranes. The inhibition curves are shown in figs. 28 & 29.

The I.C.$_{50}$'s were calculated from Hill plots of the data. (I.C.$_{50}$ = the concentration of a ligand required to give 50% inhibition of the binding).

Ligand	I.C.$_{50}$ (uM)	Hill no.
Ptx	1.87 ± 0.2	1.38 n = 2
GABA	121 ± 6	0.59 n = 2

5.3 Investigation of Chloride Flux in Locust Ganglia Tissue Preparations

5.3.1 ^{36}Chloride flux into microsacs prepared using method 4.8.1

Figure 30a) shows initial experiments to demonstrate that GABA stimulated $^{36}Cl^-$ flux could be measured in such microsac preparations from locust ganglia. GABA stimulation of $^{36}Cl^-$ flux was observed, which showed:

1) An increase with incubation time,

2) An increase with increasing concentration of GABA,

3) A reduction to control levels in the presence of 0.1 mM Triton X100.

Figure 30b shows further investigation of the dependence of

the GABA stimulation ($I.C._{50}$) = 4 μM. Figure 33, shows the dose/response curve for GABA stimulation of $^{36}Cl^-$ flux. The concentration of GABA required to give 50% of the maximum response ($E.C._{50}$) = 0.8 μM.

5.3.3 ^{36}Chloride flux into whole locust ganglia

Figure 34, shows the time course for $^{36}Cl^-$ flux into whole locust ganglia ± 0.5 mM isoguvacine.

The isoguvacine stimulated Cl^- flux increases more slowly in whole ganglia than in in microsac preparations, reaching a plateau at 30 min. Additionally in whole ganglia there was significant flux in the control tubes (in the absence of any externally added agonist) which also reached a plateau at about 30 min. Table 6, shows the effect of TBPS and Ptx on isoguvacine stimulated $^{36}Cl^-$ flux into whole locust ganglia. TBPS was able to inhibit the isoguvacine stimulation of Cl^- flux, however Ptx did not appear to inhibit the flux. Using the Mann-Whitney U test the data for TBPS was significantly different from that in the presence of isoguvacine, whilst that for pocrotoxinin was not significantly different.

Figure 7: A typical plot of total (■), non-specific (□) and specific (●) [^{35}S]TBPS binding (20 nM) to variable concentrations of locust ganglia membrane preparation. The standard deviation (sd) was < 3%, n=3. The line for non-specific binding was fitted by linear regression, whilst curves for total and specific were fitted by eye.

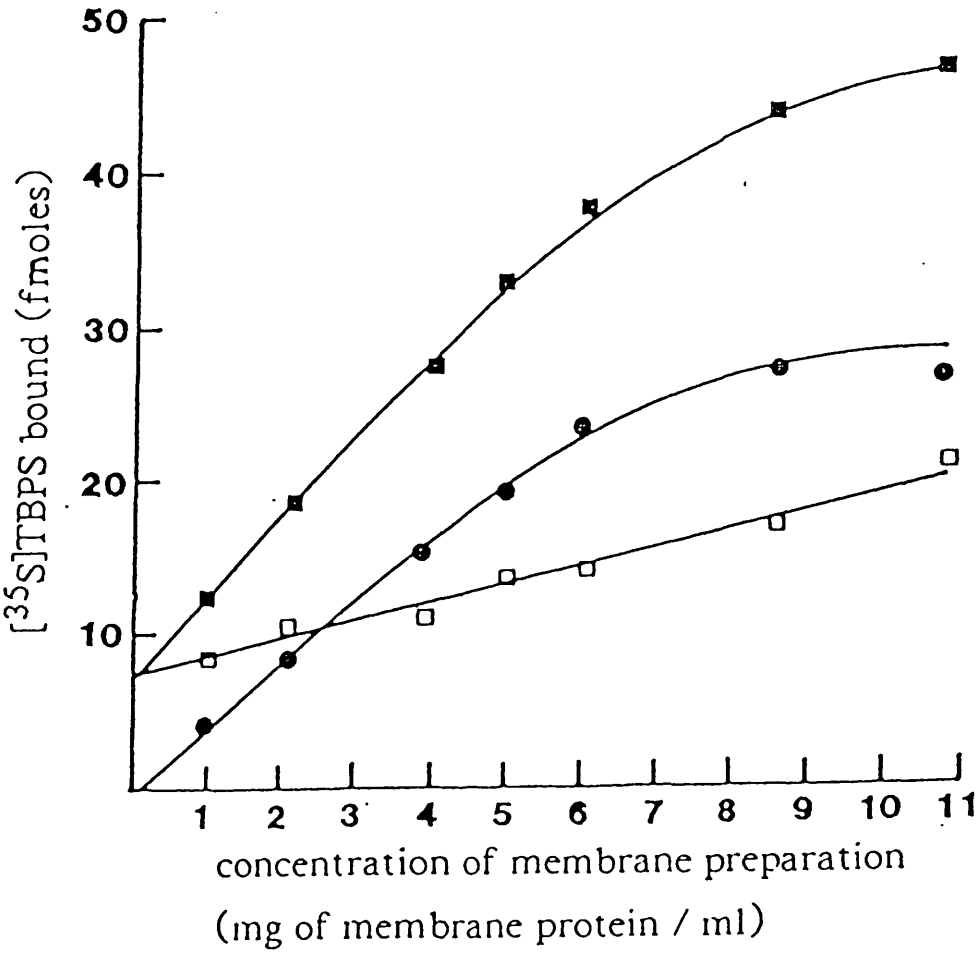


Figure 8: The pH dependence of specific [^{35}S]TBPS binding (20 nM) to locust ganglia membranes. The pH range was produced using: Tris-acetate, pH 5.0; NaH_2PO_4, pH 6.4-7.5; Tris-HCl, pH 8.0-9.5; all at 10 mM. The points are the means of 4 or more experiments, employing triplicate assays. The bars indicate the standard error of the mean (SEM). The curve was fitted by eye.

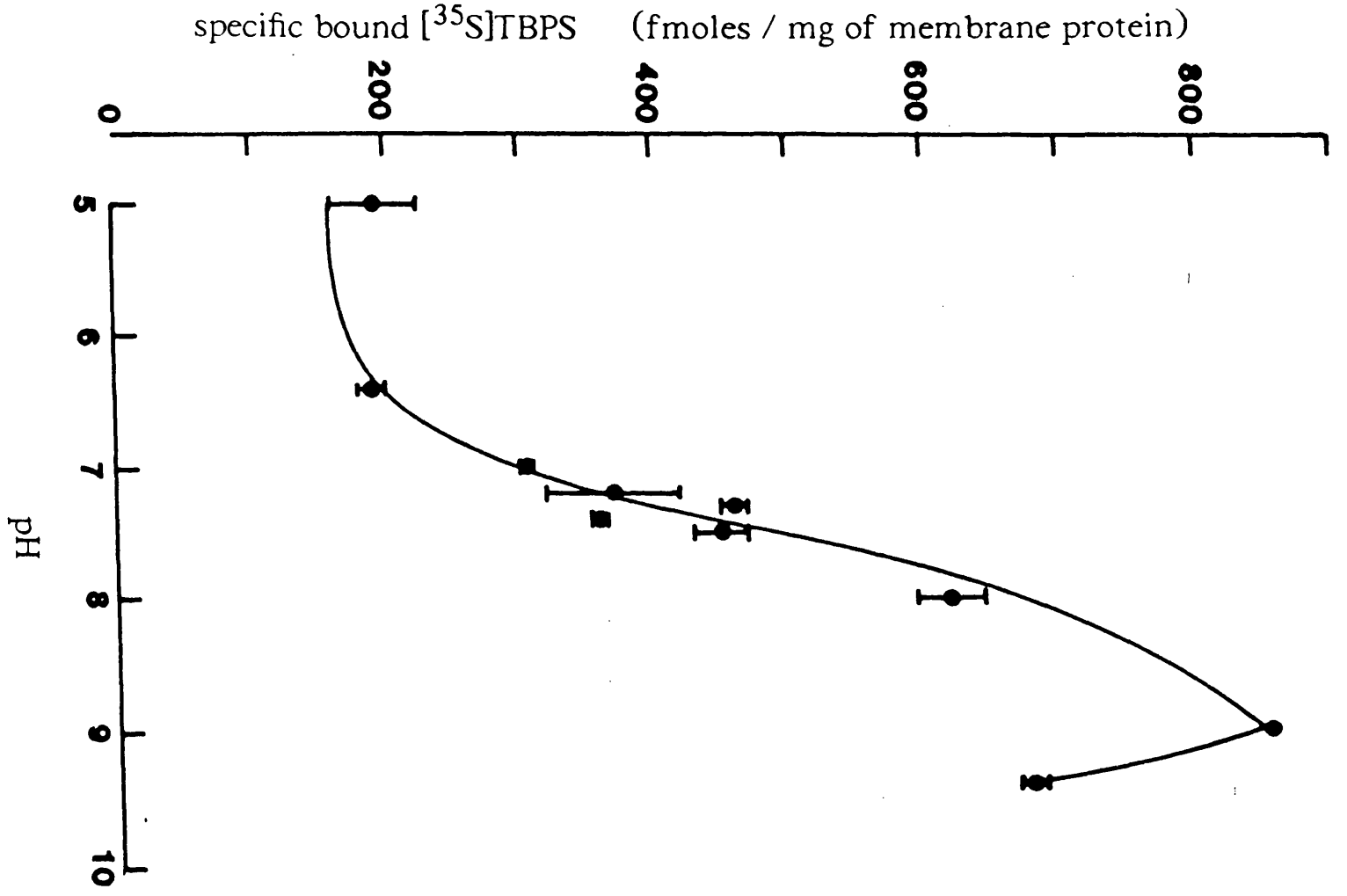

specific bound [35S]TBPS (fmoles / mg of membrane protein)
0
200
400
600
800
pH
5
6
7
8
9
10

Figure 9: A typical plot of the Cl^- dependence of specific [^{35}S]TBPS binding (20 nM) to locust ganglia membranes. The sd was 2%, n=3. The line was fitted by linear regression.
Binding in the presence of Na_2SO_4 (upto 200 mM) stayed at around 15 fmoles/mg of membrane protein (not shown).

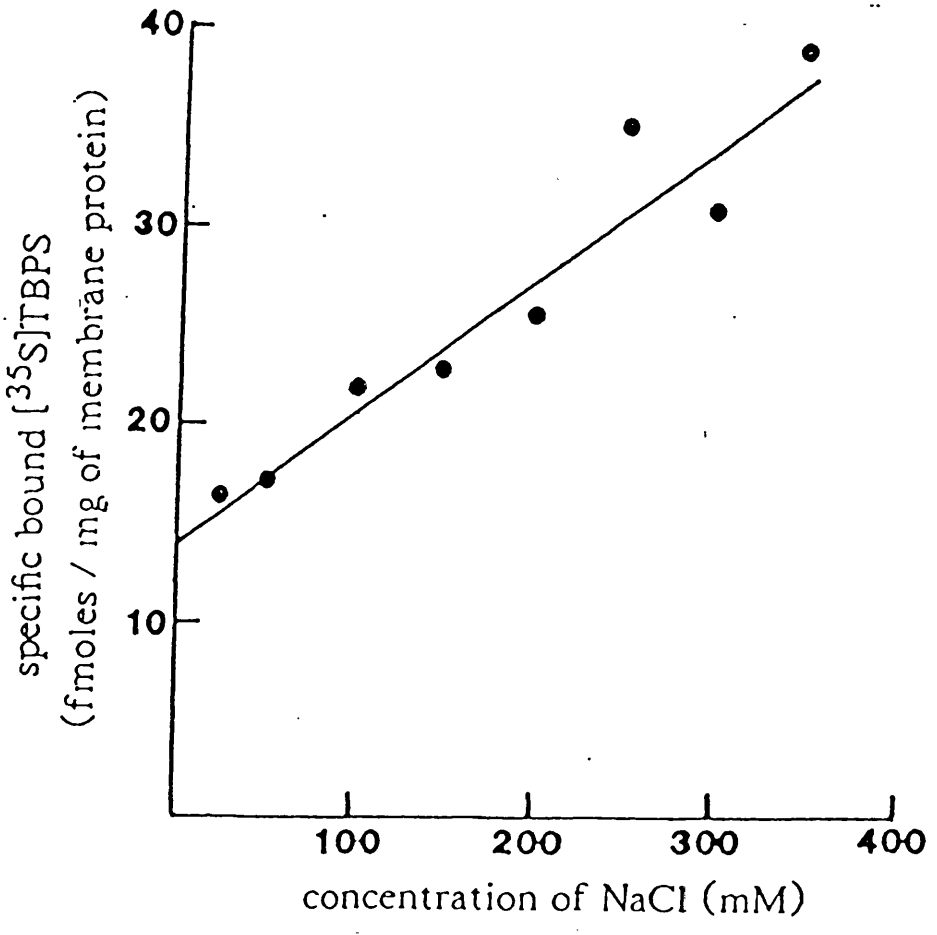
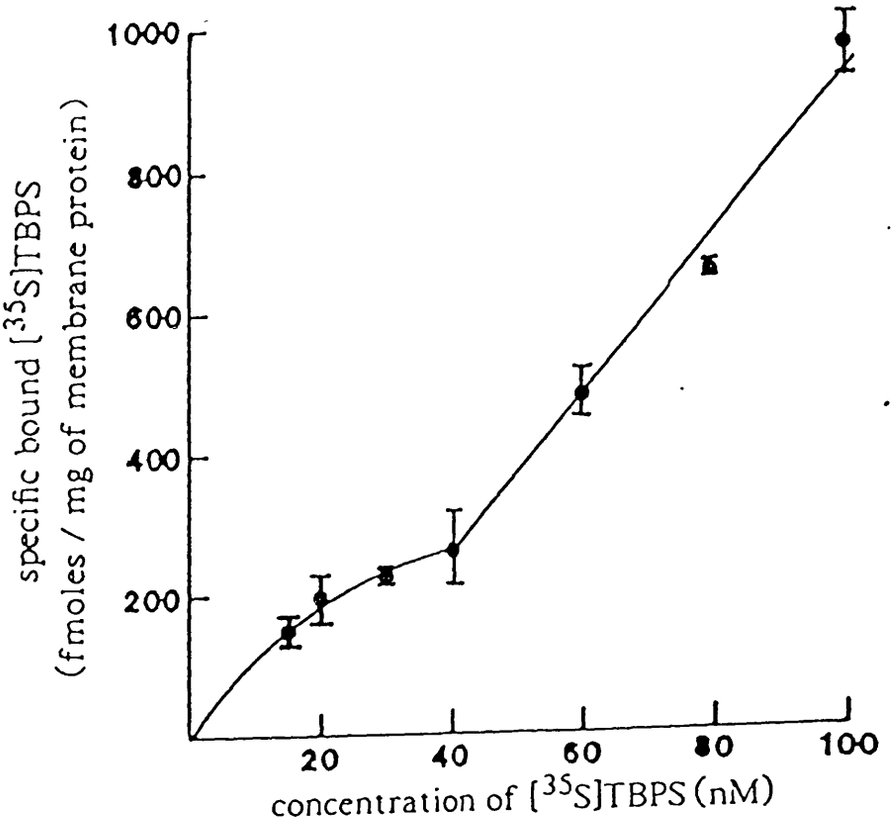


Figure 10: Binding isotherms for specific [^{35}S]TBPS binding to locust ganglia membranes.

a) The mean of 3 experiments over the range 15 - 100 nM [^{35}S]TBPS. The bars indicate the sd.

b) The mean of 3 experiments over the range 2 - 30 nM [^{35}S]TBPS + two points from (a). The bars indicate the sd.

a)


1000
800
600
400
200
specific bound [35S]TBPS
(fmoles / mg of membrane protein)
20
40
60
80
100
concentration of [35S]TBPS (nM)

b)

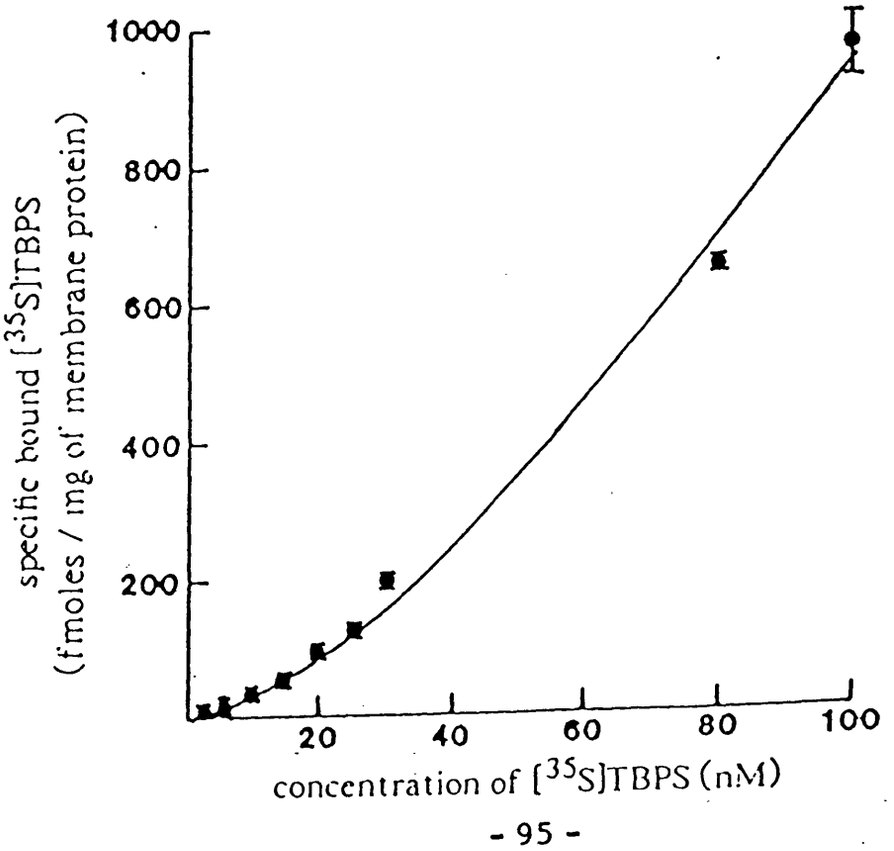

1000
800
600
400
200
specific bound [35S]TBPS
(fmoles / mg of membrane protein)
20
40
60
80
100
concentration of [35S]TBPS (nM)

Figure 11: Scatchard analysis of the combined data from 6 experiments represented by fig. 10. The curve was fitted by eye.

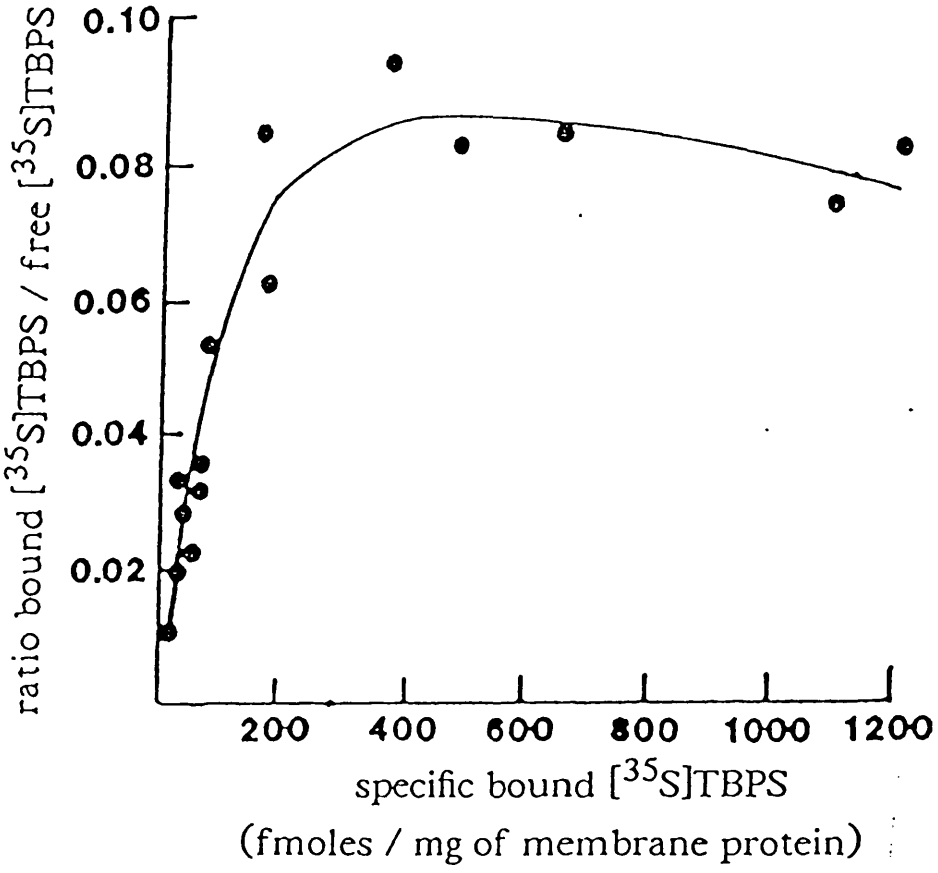


Figure 12: Hill analysis of the data in fig. 11. The line was fitted by eye.

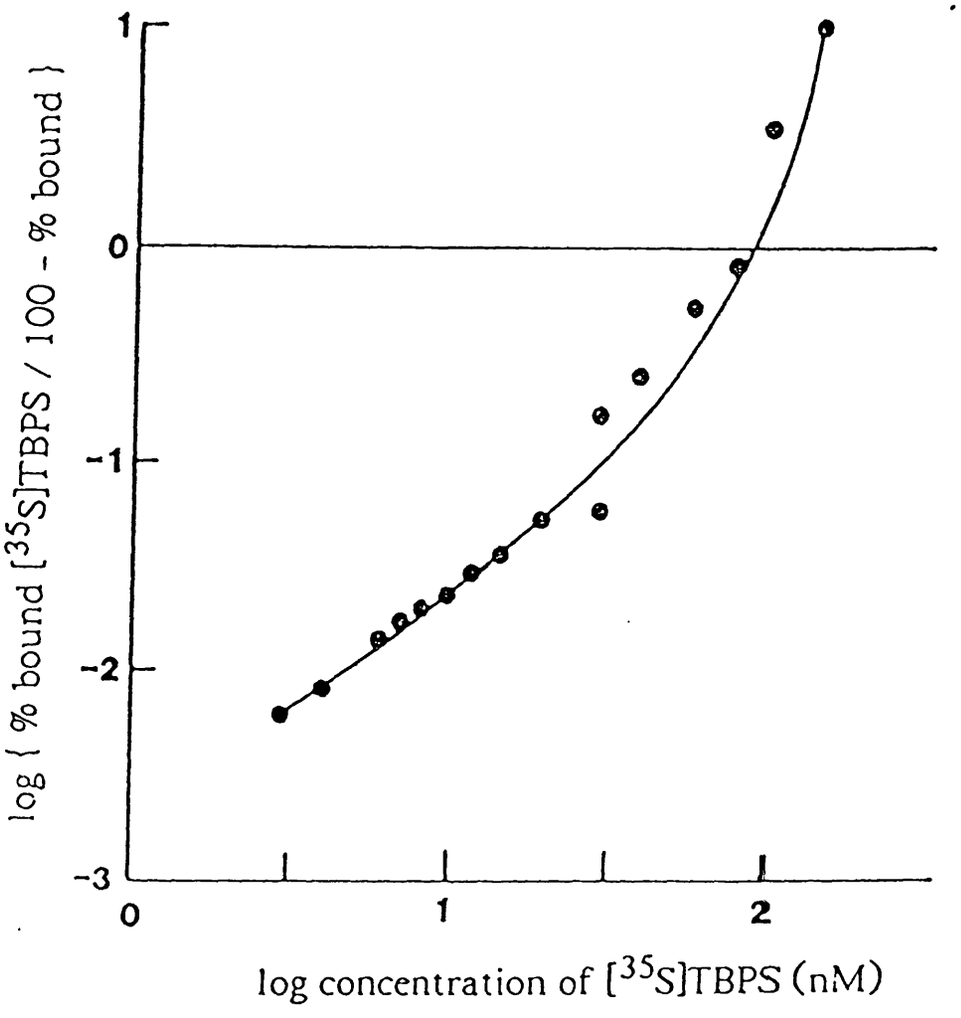


Figure 14: Binding isotherm for the higher concentration range of specific [^{35}S]TBPS, (10 - 600 nM) binding to locust ganglia membranes. The bars indicate the sd, n=3.. The curve was fitted by eye.

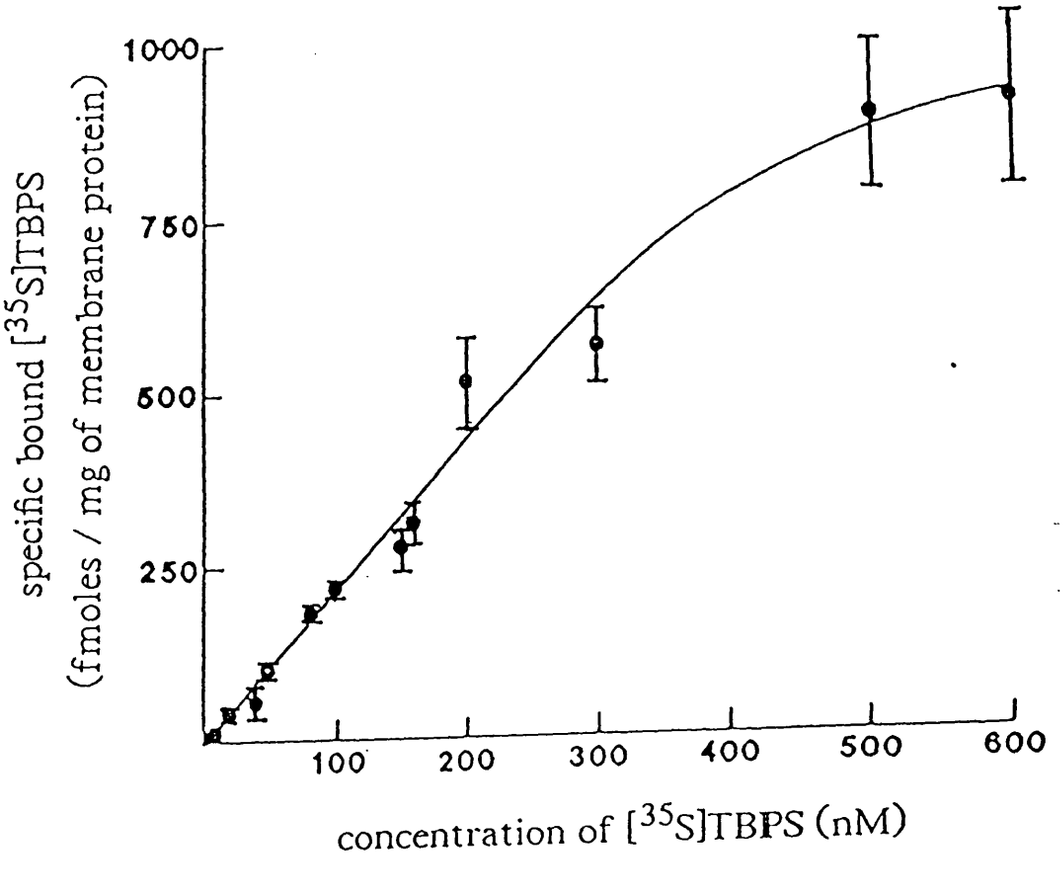


Figure 15: Plot of ln specific bound [^{35}S]TBPS at equilibrium (B_{eqm}) divided by B_{eqm} minus bound at time, t (B_t) versus time: for the association of 12 (□) and 21 (■) nM [^{35}S]TBPS. Each point is the mean of 3 experiments. The bars indicate the error (expressed as the percentage error that the sd was, of the original data before it was transformed.

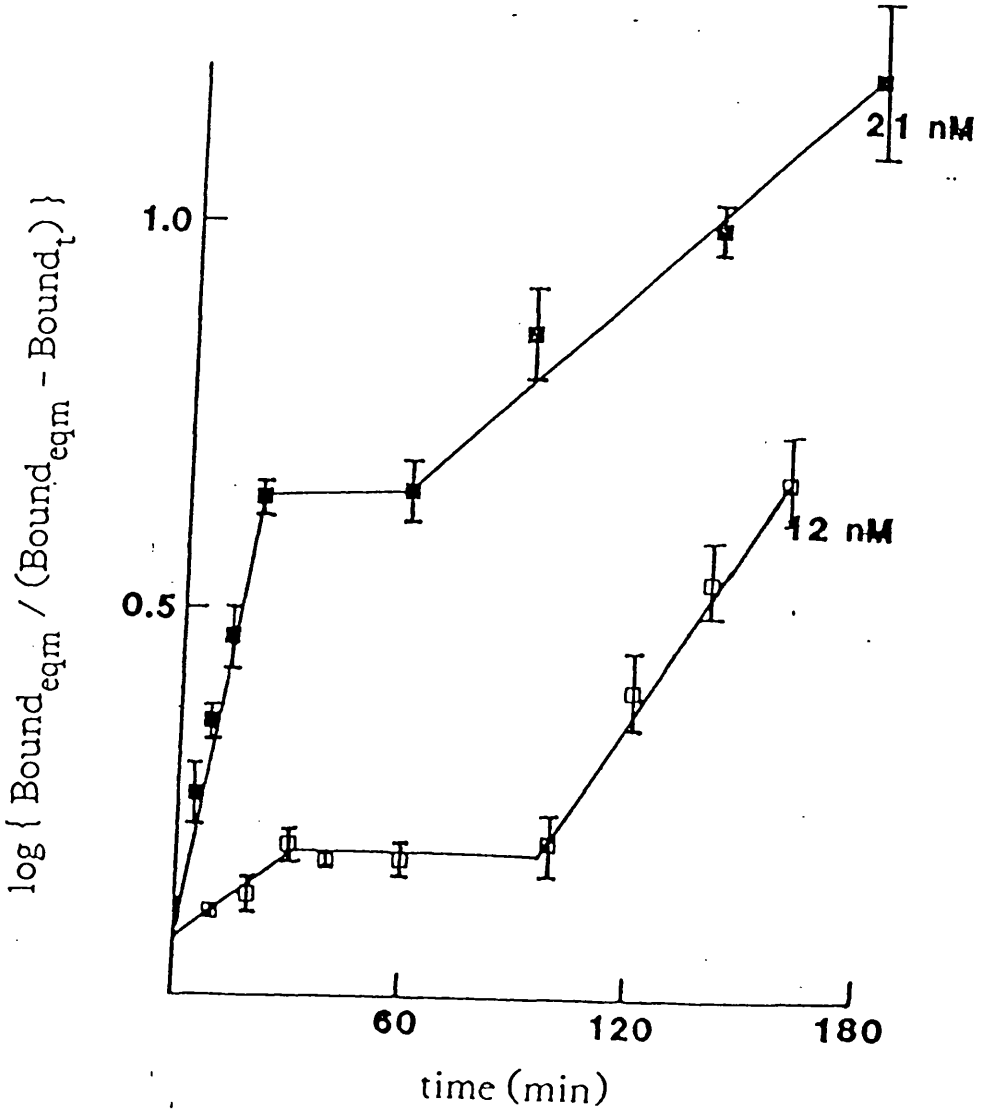


Figure 16: The same as fig. 15 but for 32 (□) and 42 (■) nM [^{35}S]TBPS. The bars indicating the error were also calculated in the same manner.

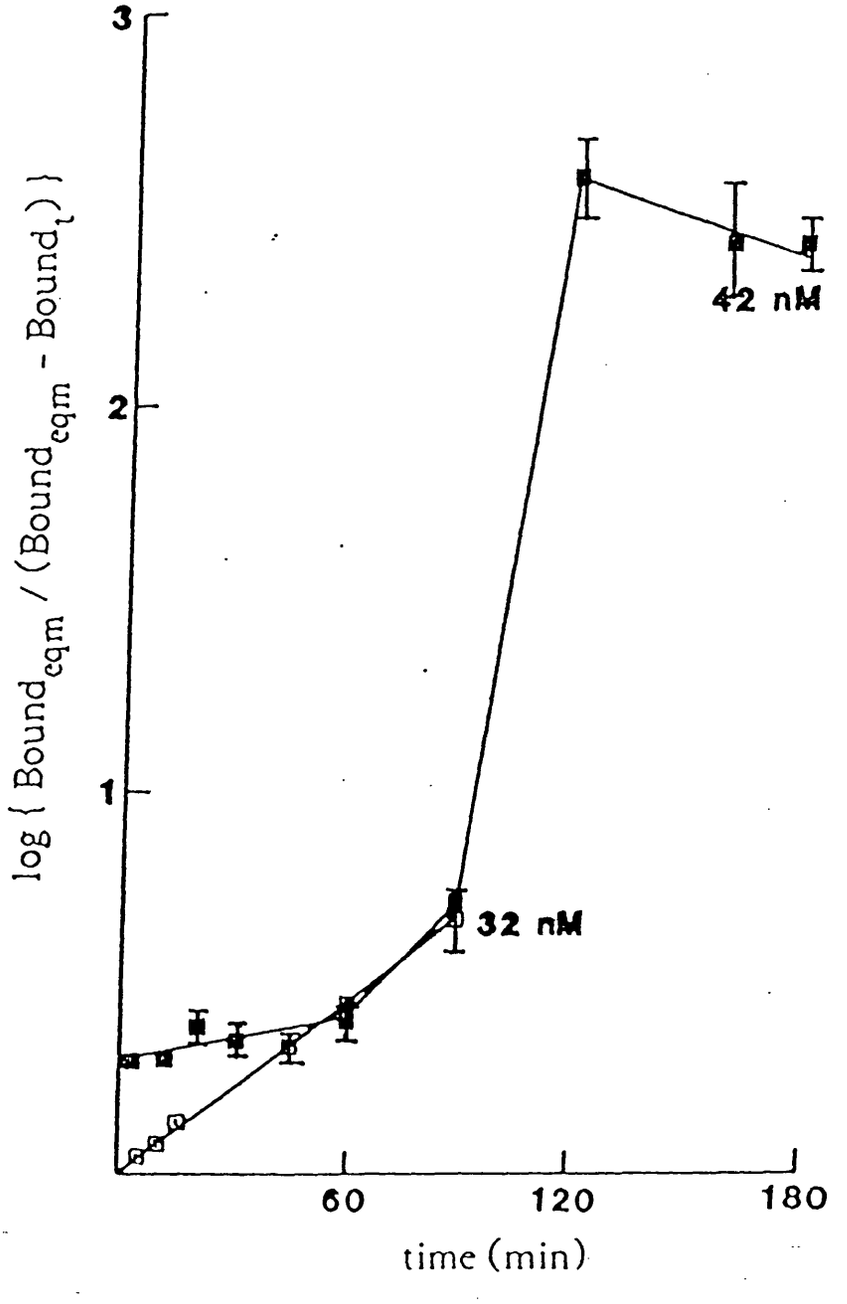


Figure 17: To further emphasise the non-linear nature of the association of [^{35}S]TBPS the secondary plot of k_{1obs} versus concentration of [^{35}S]TBPS was constructed. The two lines represent the gradients of the apparent early (■) and late (□) phases of association shown in figs. 15 & 16. These gradients are tabulated in table 3.

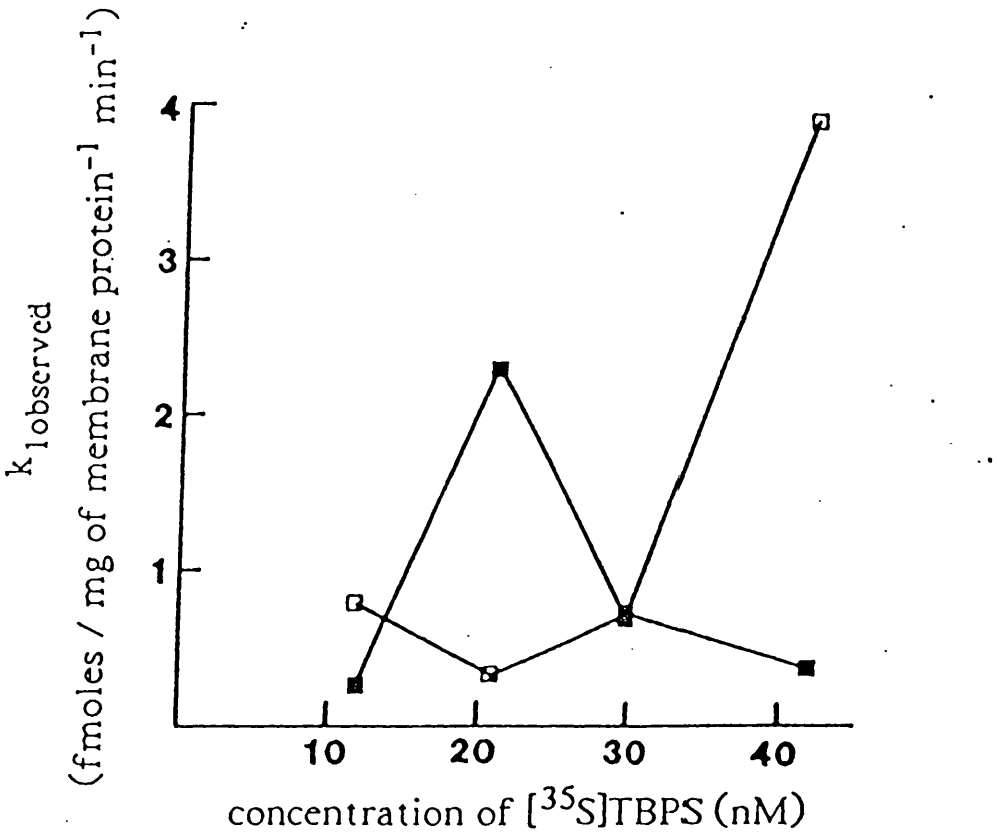


Figure 18: Plot of specific bound [^{35}S]TBPS to locust ganglia membranes versus the concentration of unlabelled TBPS which was preincubated with the membranes for 2 hours. This is a typical experiment of 2. The line was fitted by eye.

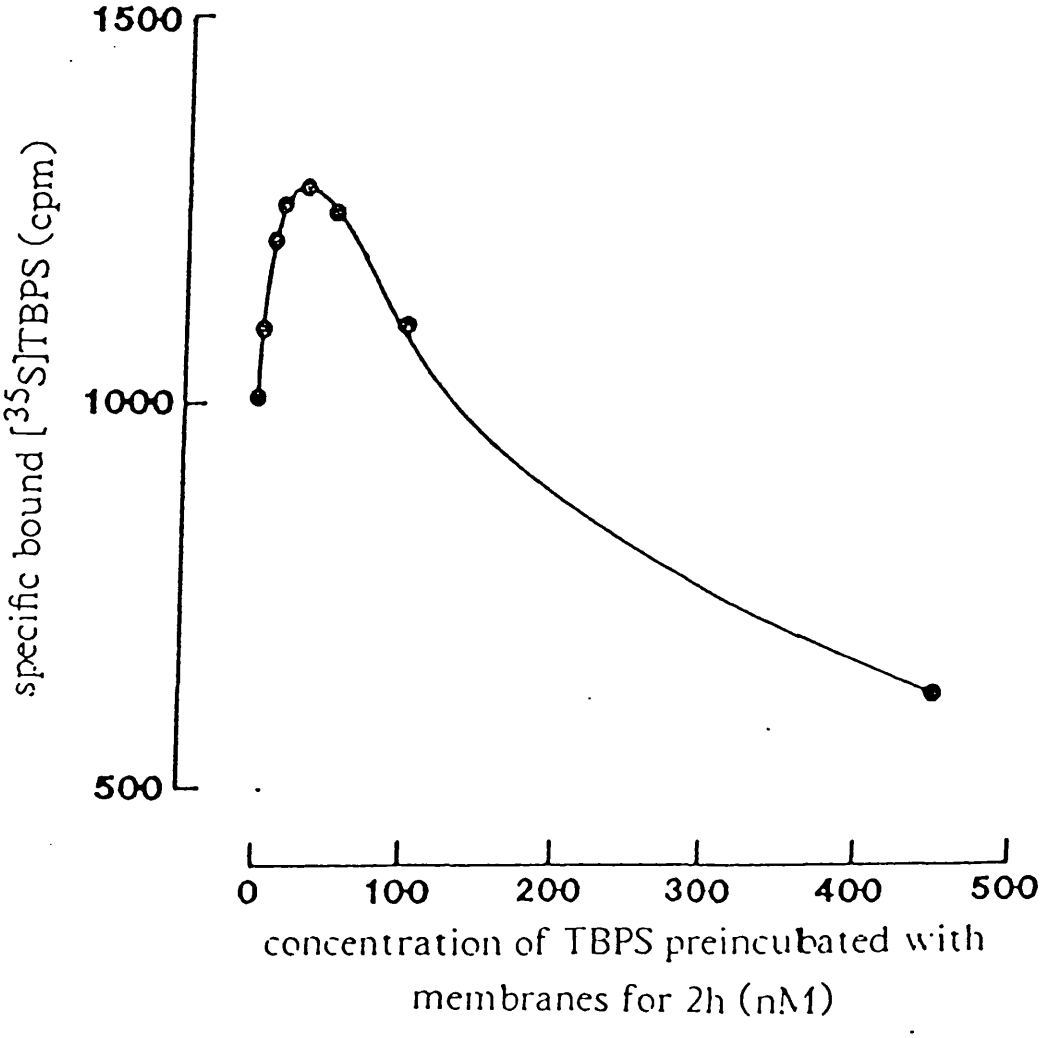


Figure 19: Plot of % bound [^{35}S]TBPS at time zero (%B) versus time for the dissociation of 20 nM [^{35}S]TBPS (at equilibrium, 3h) initiated by; 45 mM unlabelled TBPS (■) and 100 fold dilution with assay buffer (□). The points for each line are the mean of 3 experiments. The bars indicate the sd of the %B. The lines were fitted by eye.

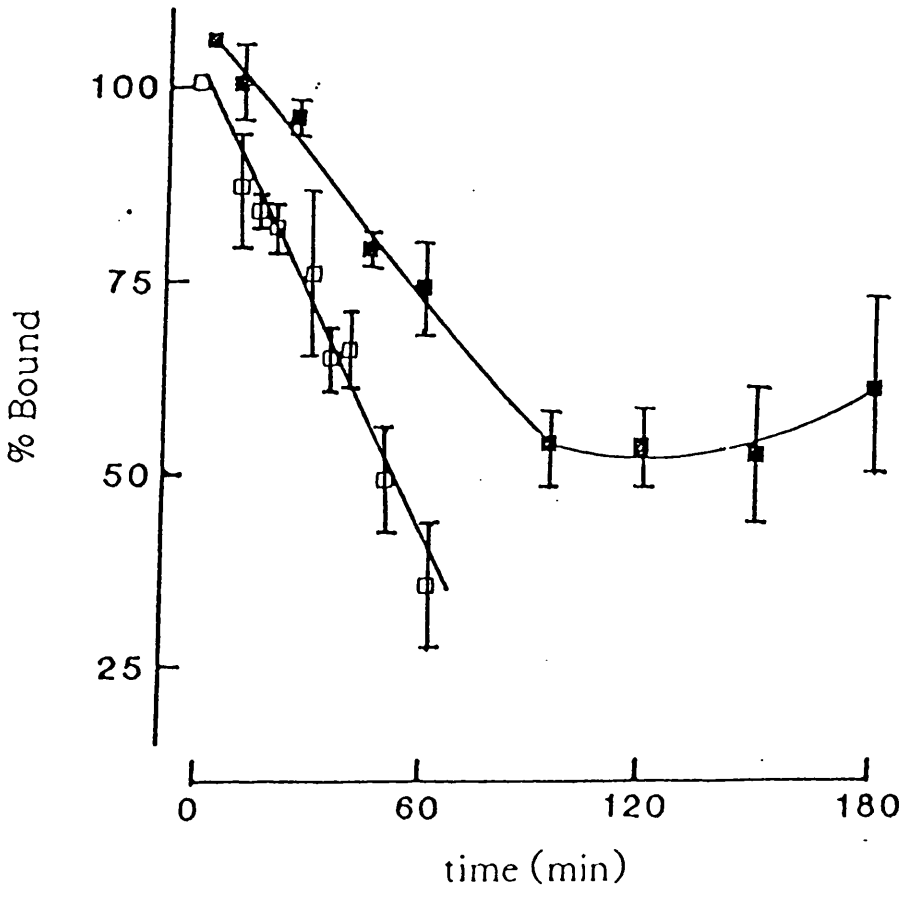


Figure 20: Plot of natural log (ln) concentration of bound [^{35}S]TBPS at time, t (B_t) over concentration of [^{35}S]TBPS at time zero (B_0) versus time; for the dissociation of 20 nM [^{35}S]TBPS (at equilibrium) initiated by 100 fold dilution with assay buffer. The data for the points was the same as that used in fig. 19. The line was fitted by eye and is initially linear.

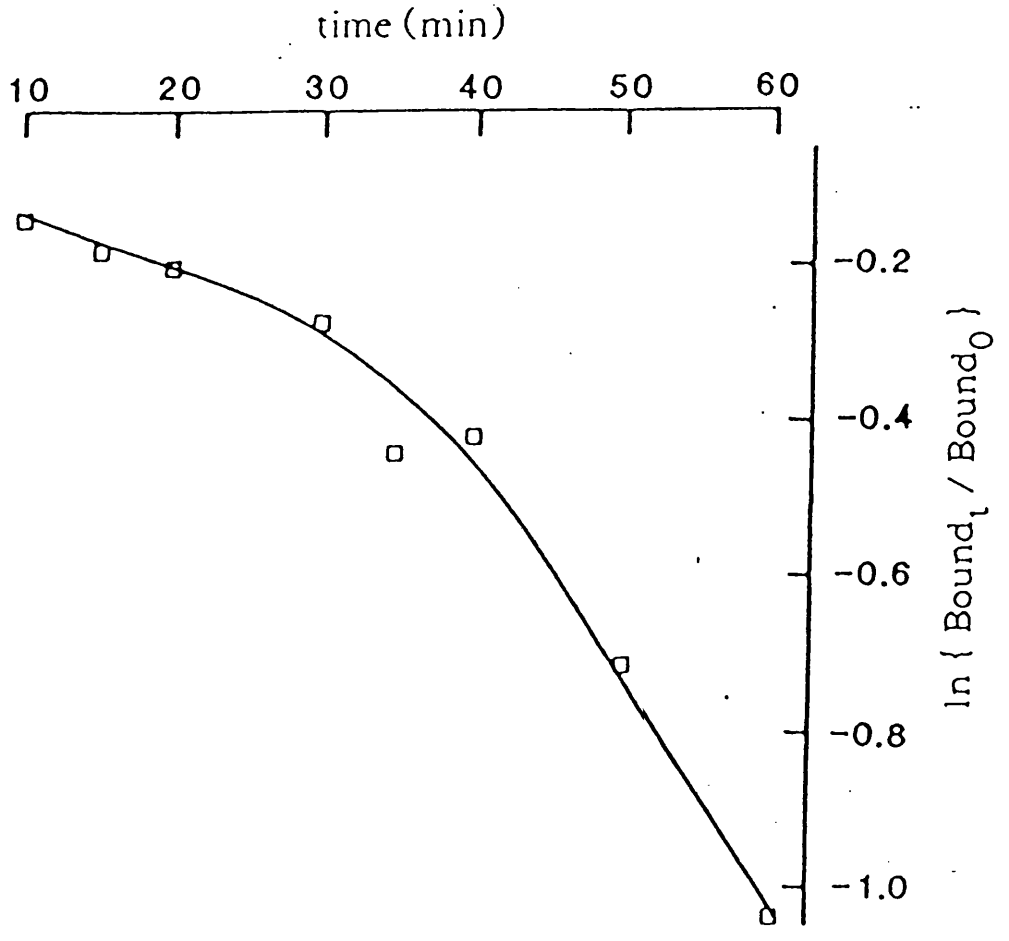


Figure 21: Plot of ln (B_t / B_0) versus time; for dissociation of 20 nM [^{35}S]TBPS (at equilibrium), initiated by 45 mM unlabelled TBPS. The data was the same as that used in fig. 19. The line was fitted by eye, and although it was initially linear, reaches a plateau after 40 min.

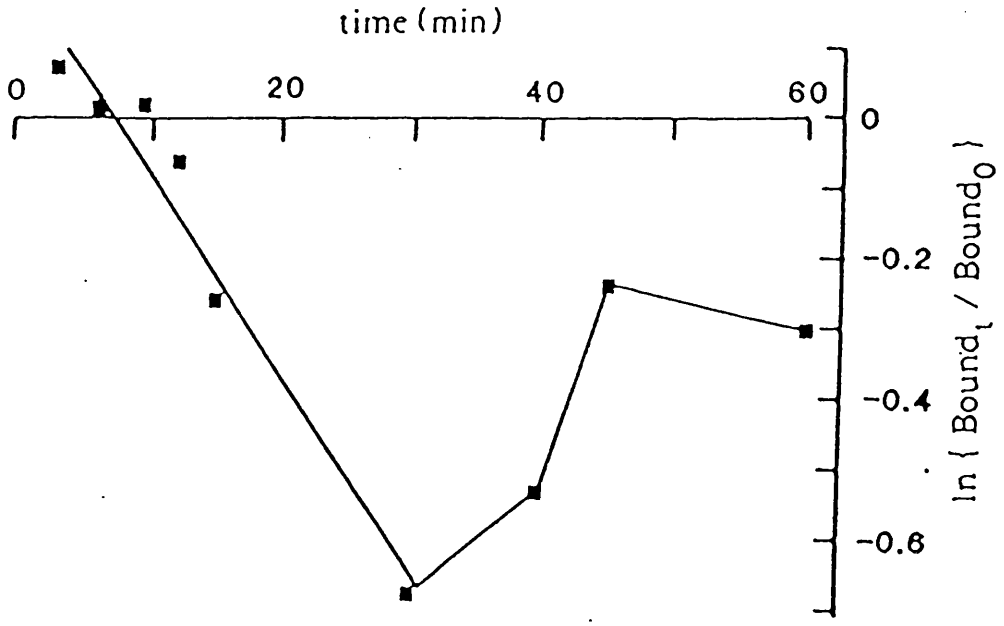


Figure 13: Scatchard analysis of a restricted concentration range of [^{35}S]TBPS binding to a higher concentration of locust ganglia membranes. A typical plot of two. The line was fitted using linear regression.

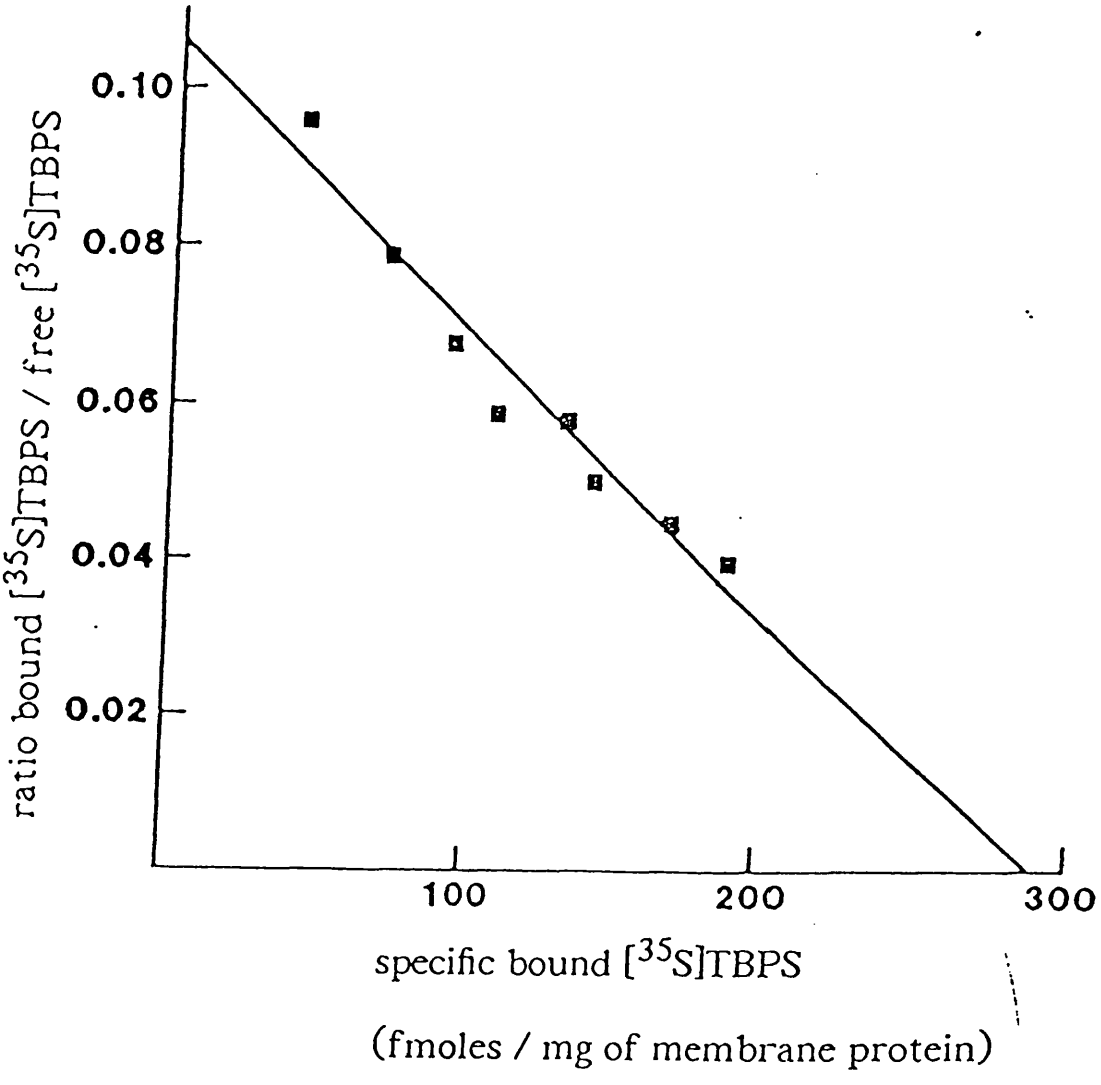


Figure 22: The effect of TBPS (■) and IBP (□) on the binding of 20 nM [^{35}S]TBPS to locust ganglia membranes. The TBPS curve is typical of 8 experiments and that for IBP is typical of 6 experiments. The sd was <5%. The curves were fitted by eye.

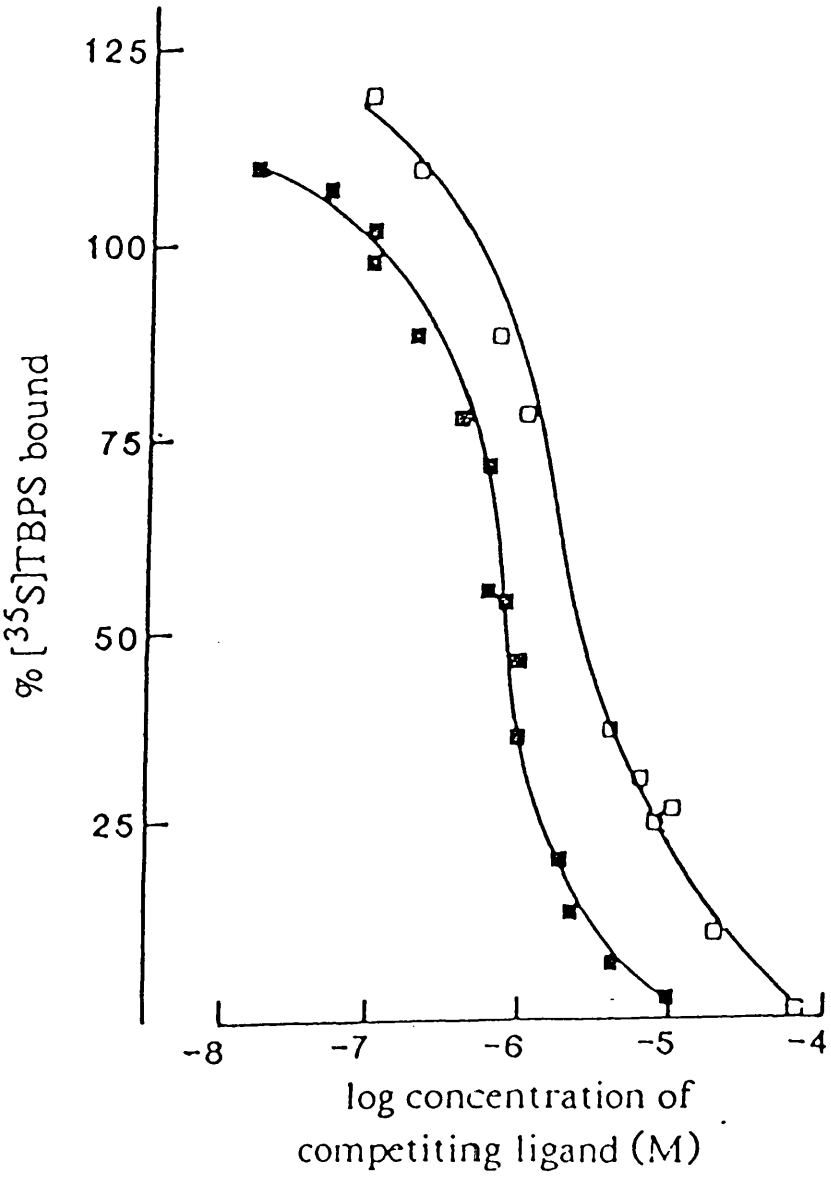


Figure 23: The effect of GABA on the binding of 20 nM [^{35}S]TBPS to locust ganglia membranes. The curve was fitted by eye and is typical of 3 experiments. The sd was <5%.

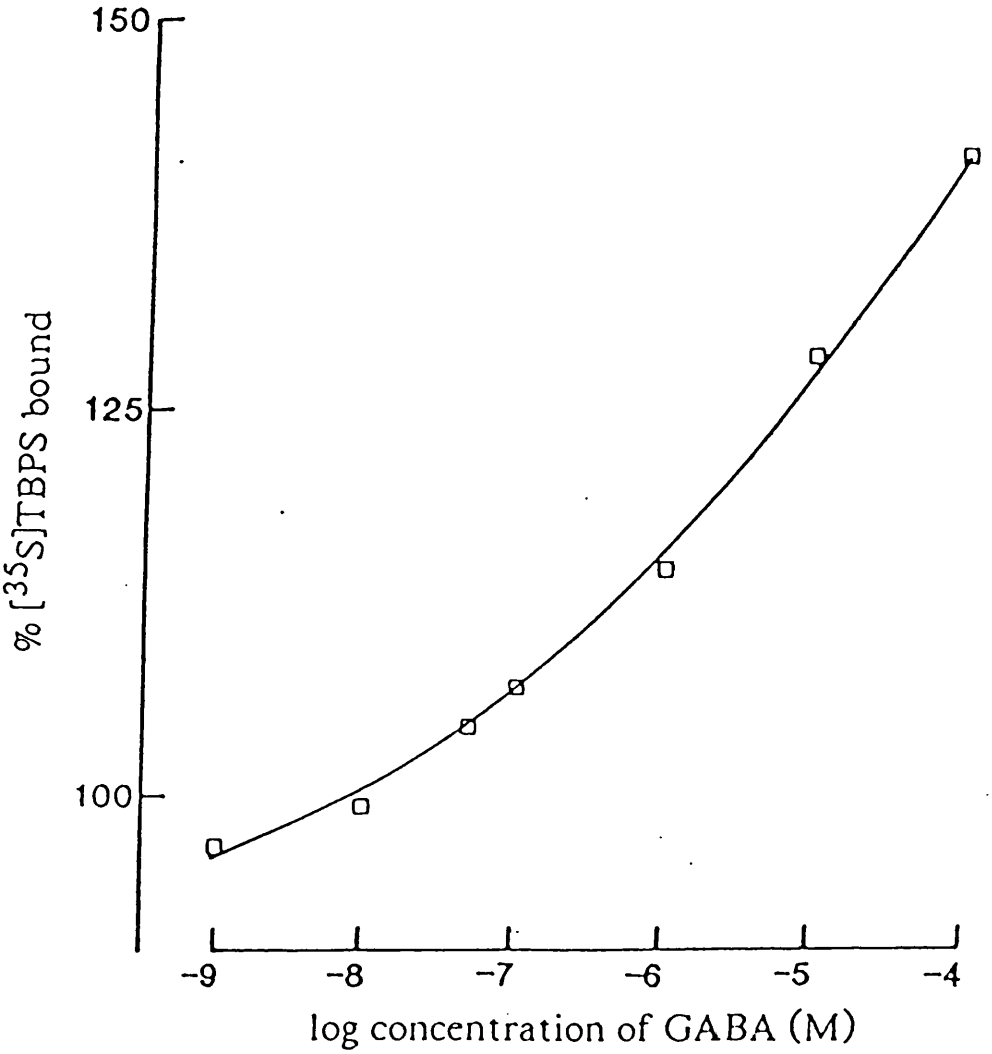


Figure 24: The effect of two benzodiazepines on the binding of 20 nM [^{35}S]TBPS to locust ganglia membranes. The curve for clonazepam (□) is typical of 3 experiments, whilst that for Ro5-4864 (■) is typical of 5 experiments. The curves were fitted by eye. The sd was <5%.

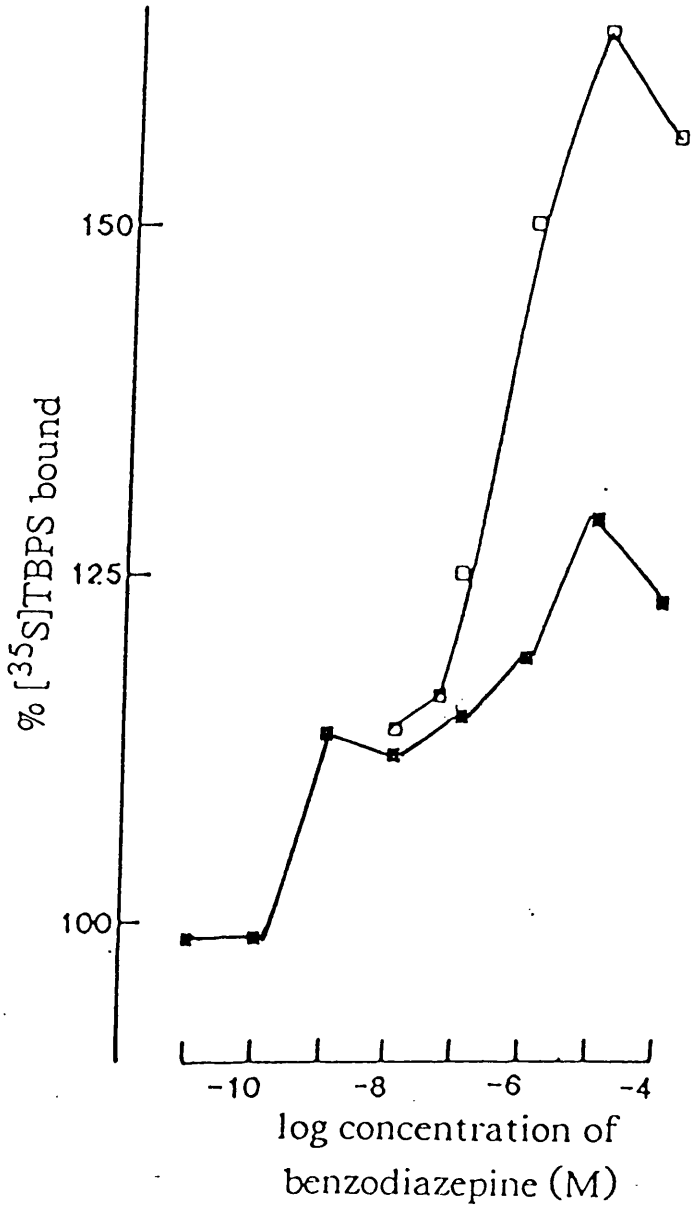


Figure 25: The effect of pentobarbital on the binding of 20 nM [^{35}S]TBPS to locust ganglia membranes. The curve was fitted by eye and is typical of 2 experiments. The sd was <5%.

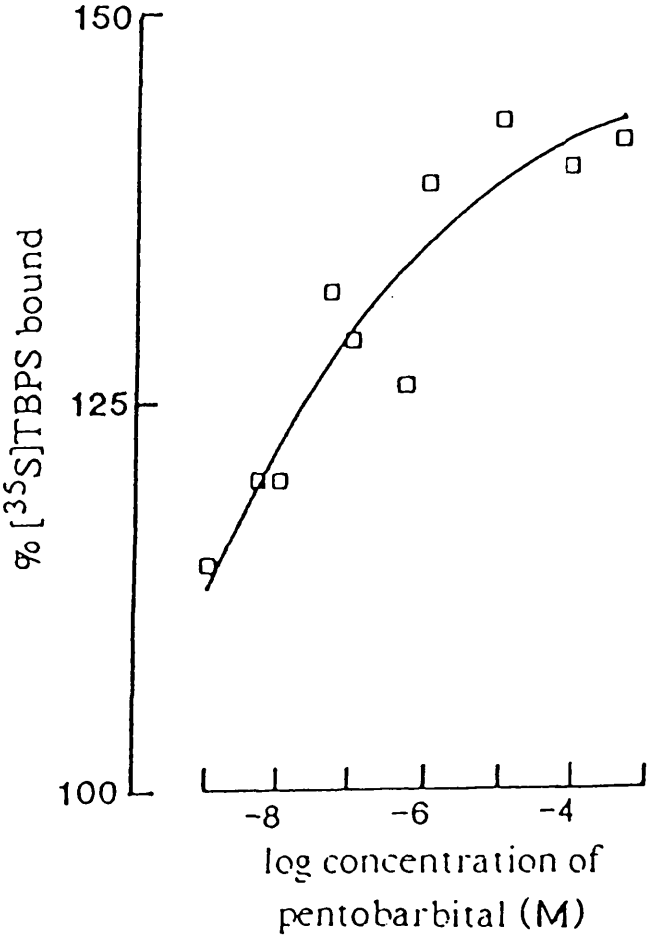


Figure 26: The effect of insecticides on the binding of 20 nM [^{35}S]TBPS to locust ganglia membranes. The curve for cypermethrin (▲) and dieldrin (●) are typical of 3 experiments whilst that for lindane (■) is typical of 6 experiments. Lindane was also found to enhance the binding, data not shown. The curves were fitted by eye. The sd was <5%.

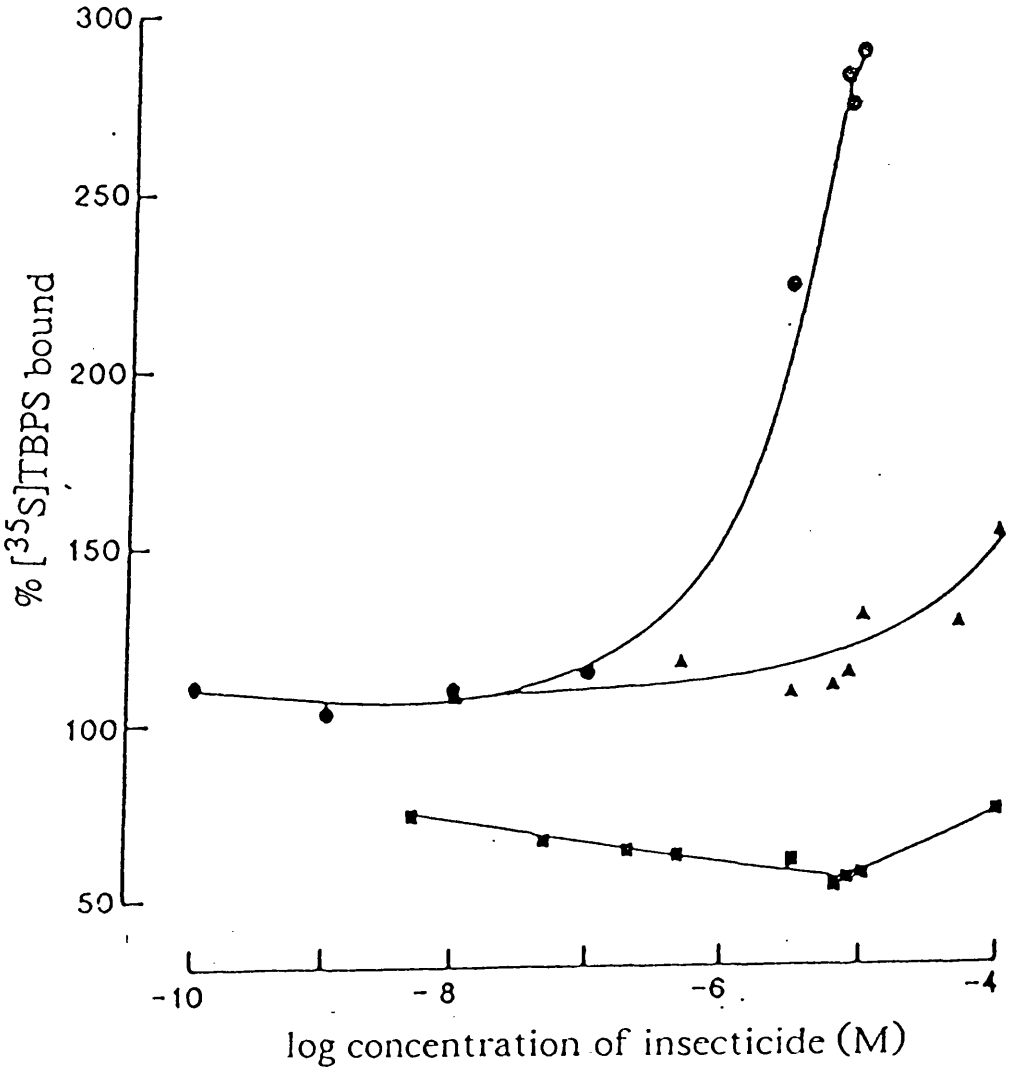


Figure 27: The binding isotherm for the specific binding of [^{35}S]TBPS to rat brain membranes. The points are the mean of 3 experiments and the bars indicate the sd. The curve was fitted by eye.

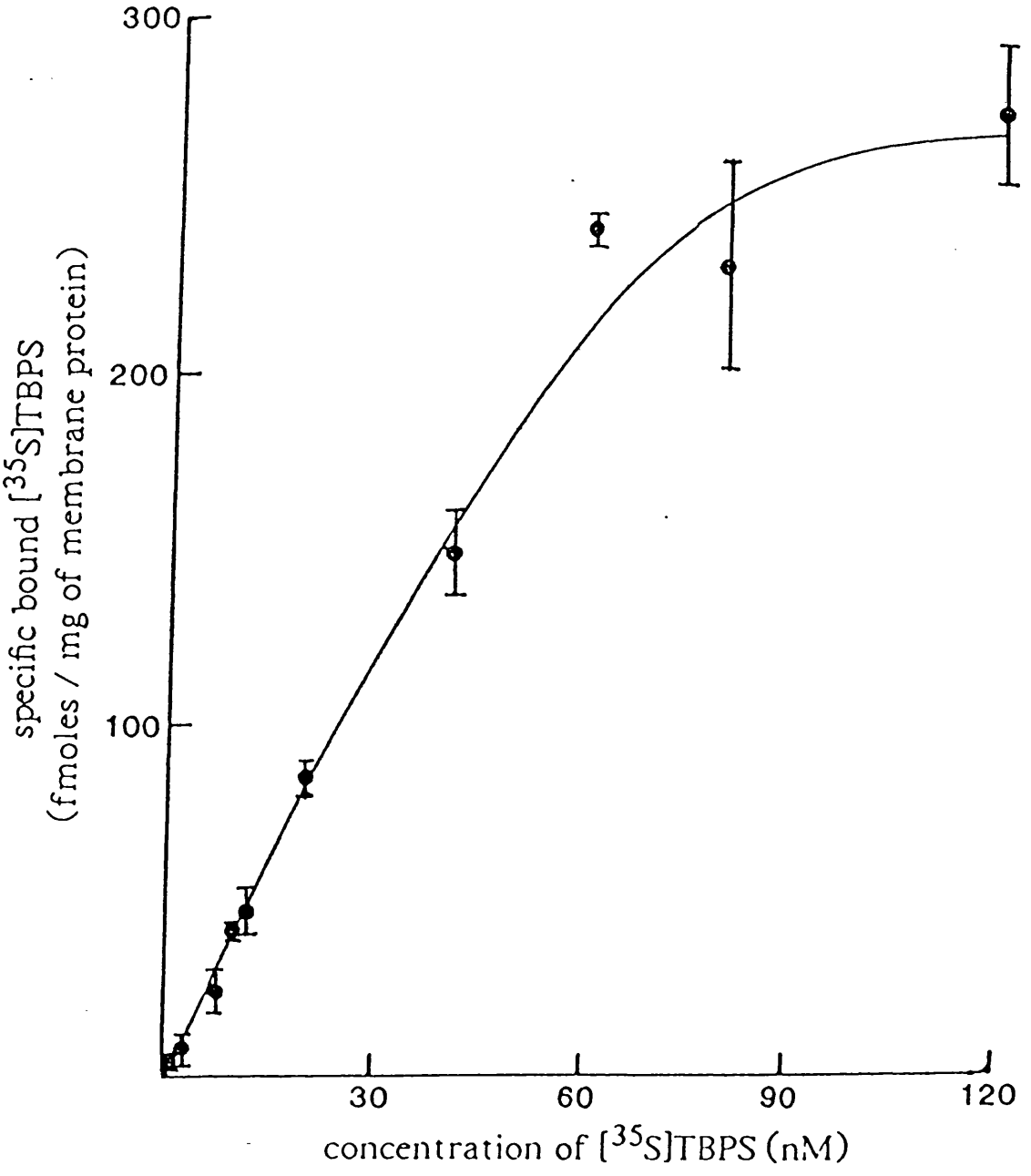


Figure 28: The effect of picrotoxinin on the specific binding of 50 nM [^{35}S]TBPS to rat brain membranes. The curve was fitted by eye and is typical of 2 experiments. The sd was 5-10%.

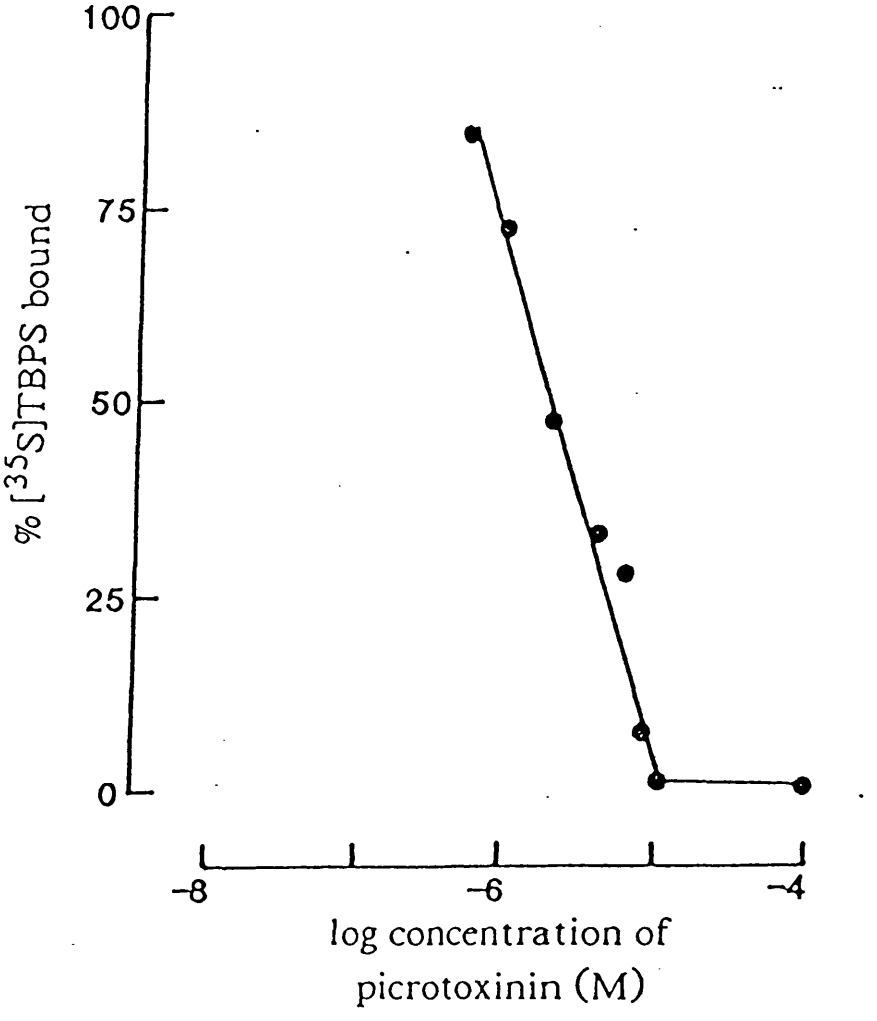


Figure 29: The effect of GABA on the specific binding of 50 nM [^{35}S]TBPS to rat brain membranes. The curve was fitted by eye and is typical of 2 experiments. The sd was 8%.

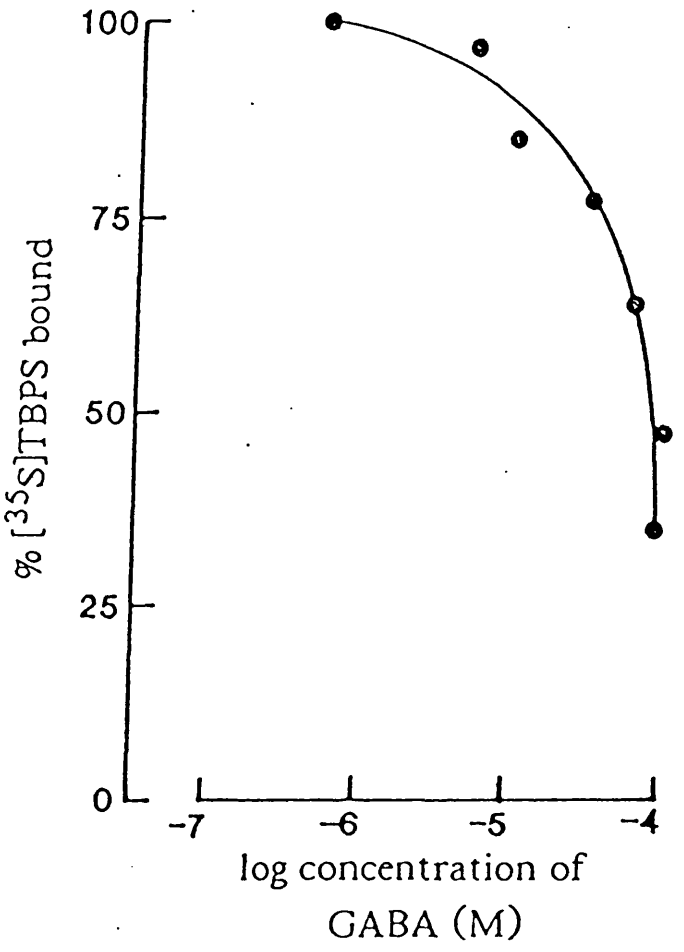


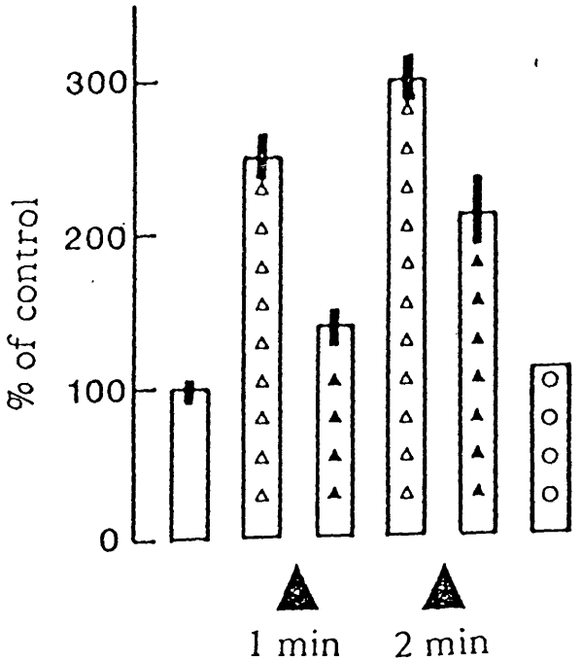
Figure 30:

a) A histogram to show the effect of 0.1 mM (△) and 1 μM (▲) GABA, triton X100 and time on $^{36}Cl^-$ flux in locust ganglia microsacs. The bars indicate the SEM.

b) $^{36}Cl^-$ flux in locust ganglia microsacs versus time: in the presence of 0.1 mM GABA (■) and the absence of any additional GABA (□). (▲) shows the difference between the two lines. The lines were fitted by eye. This is a representative plot, problems with reproducibility meant that the results from similar experiments could not be averaged.

In both (a) and (b) the microsacs were produced using the method described in 4.8.1.

a)


300
200
100
0
% of control
1 min
2 min

b)

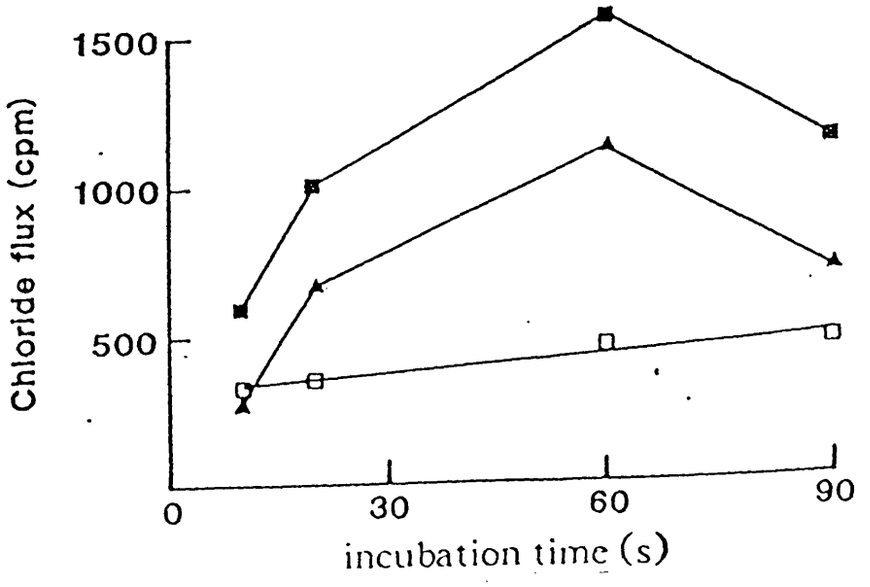

1500
1000
500
Chloride flux (cpm)
0
30
60
90
incubation time (s)

Figure 31: $^{36}Cl^-$ flux in locust ganglia microsacs (prepared by the gentler method described in 4.8.2) versus time: in the presence of 50 μM GABA (●) and the absence of any additional GABA (O). Each point is the mean of 3 experiments and the bars indicate the sd.

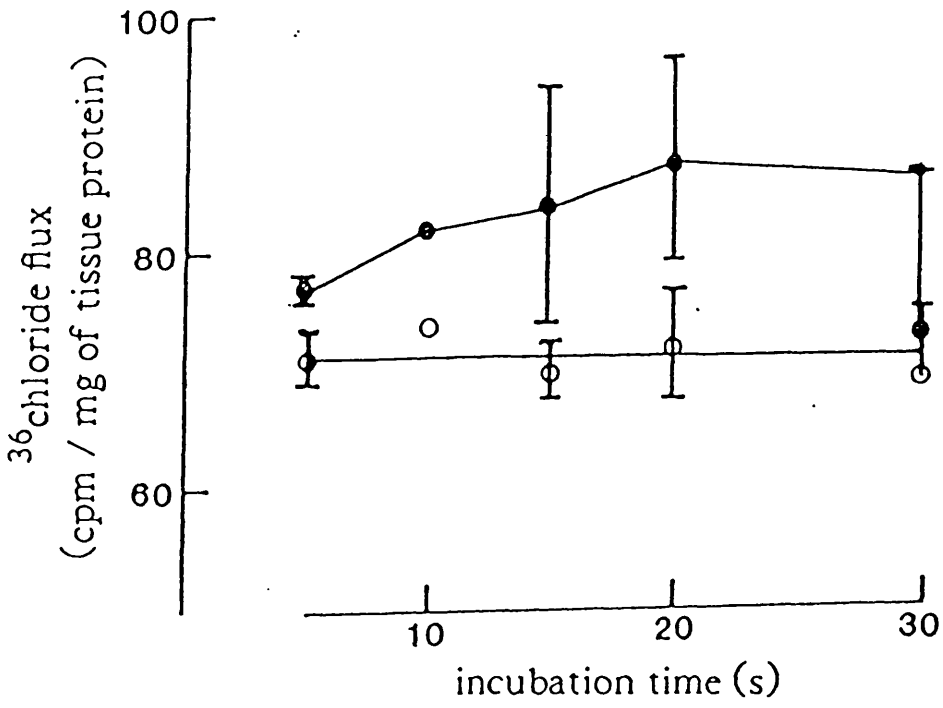


Figure 32: The effect of DIDS on the GABA stimulated $^{36}Cl^-$ flux in locust ganglia microsacs, prepared by the method described in 4.8.2.

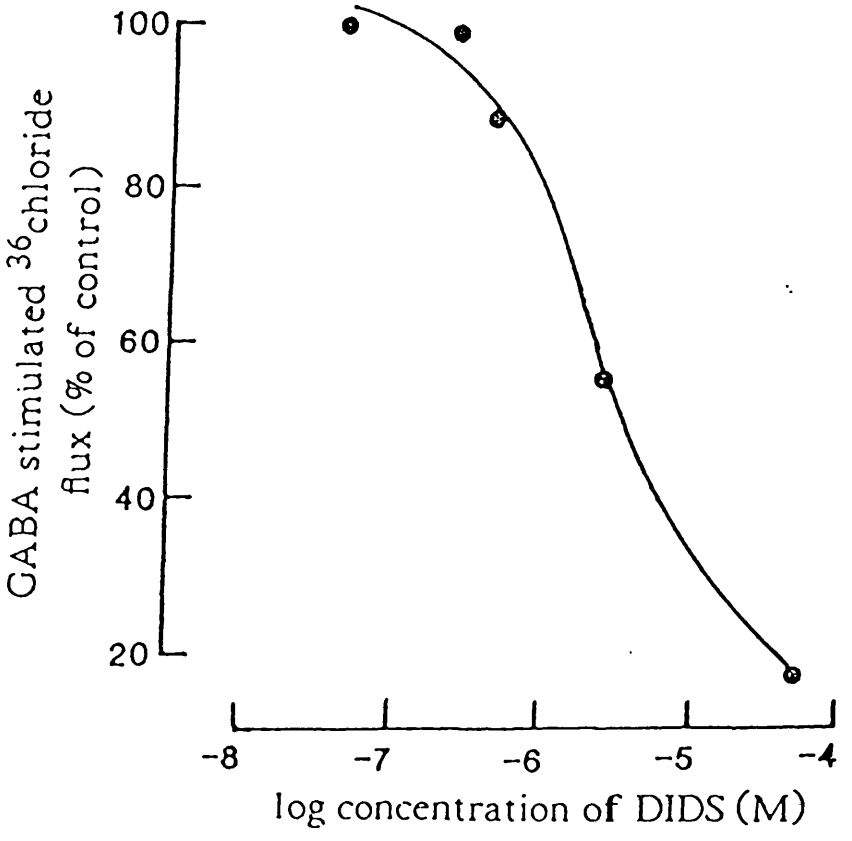


Figure 33: The effect of GABA on the $^{36}Cl^-$ flux in locust ganglia microsacs, prepared by the method described in 4.8.2. Each point is the mean of 3 experiments and the bars indicate the sd.

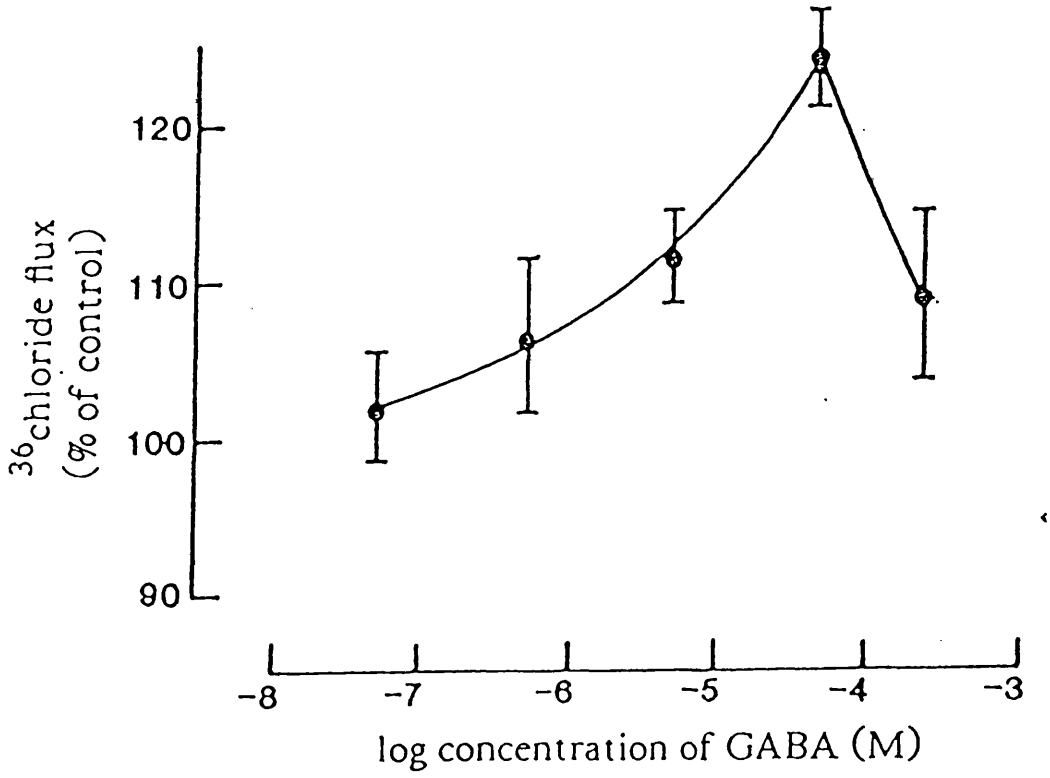


Figure 34: $^{36}Cl^-$ flux in whole locust ganglia : in the presence of 0.5 mM isoguvacine (●) and the absence of any additional agonist (■). Each point is the mean of 3 experiments and the bars indicate the sd.

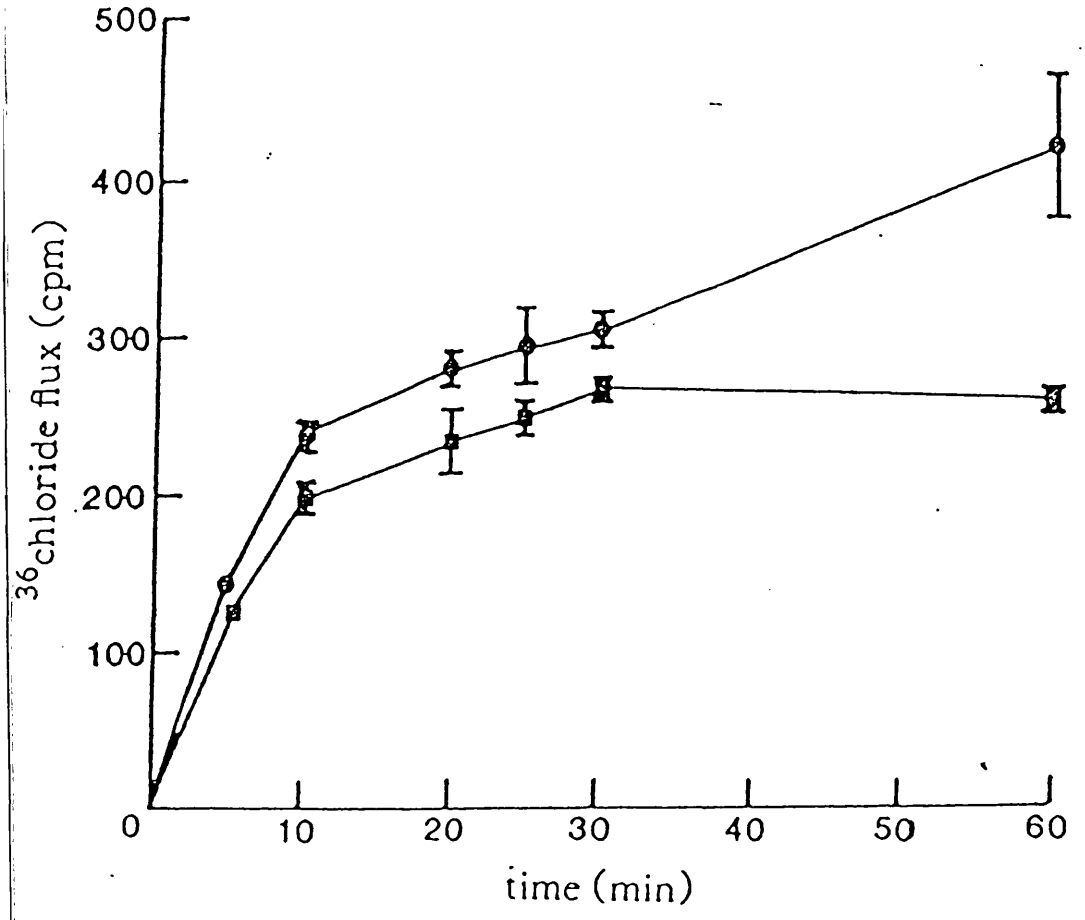


Table 3. $k_{1observed}$ values for the association of [^{35}S]TBPS to locust ganglia membranes

concentration of [^{35}S]TBPS (nM)	k_1 (early) (min^{-1})	±sd	k_1 (late) (min^{-1})	±sd
12	0.003	0.001	0.008	0.0005
21	0.023	0.001	0.003	0.0004
32	0.007	0.0003	0.007	0.0003
42	0.004	0.0006	0.039	0.015

This table contains the gradients obtained from figs. 15 & 16. Such plots should give one gradient, however 3 lines in figs. 15 & 16 give two gradients. Thus it appears that k_1 is changing and is thus not a true constant. These values were used to produce the secondary plot shown in fig. 17, which further suggests by its non-linear nature that the association rate constant, k_1 is changing with varying concentrations of TBPS and is thus not a true constant.

TABLE 4: The effect of various ligands on the binding of [^{35}S]-TBPS to locust ganglia membranes

Ligand	E.C.$_{50}$ (μM)	Maximum effect (%)
CONVULSANTS		
TBPS	0.68	-'ve 100
IBP	2.24	-'ve 100
Picrotoxinin	n.a	0
GABA agonist		
GABA	2.5	+'ve 145
BENZODIAZEPINES		
Diazepam	n.t	+'ve 130
Clonazepam	0.06	+'ve 130
Ro5-4864	0.13	+'ve 155
BARBITURATES		
Pentobarbital	0.02	+'ve 150
INSECTICIDES		
Cypermethrin	44.7	+'ve 160
Permethrin	n.a	0
Dieldrin	1.40	+'ve 310
Lindane	2.5	+'ve 175
Lindane	10	-'ve 50 (biphasic)

The EC_{50} was defined as the concentration of the ligand required to give 50% of its effect. -'ve values indicate that the ligand was inhibitory and +'ve values that the ligand was stimulatory.

n.a - no action.

n.t - not tested.

Table 5: The effect of various GABAergic agonists and antagonists on ^{36}chloride flux in locust ganglia microsacs

	^{36}chloride flux (% of control)	± s.d	n
control	100	1	7
GABA (50 μM)	120	10	7
isoguvacine (0.5 mM)	170	50	2
APS (0.5 mM)	128	4	2
	GABA (50 μM) +		
Ptx (50 μM)	123	16	5
TBPS (23 μM)	130	16	3
bicuculline (0.5 mM)	128	1	2
DIDS (2 mM)	95	3	2
water	80	1	2

The microsacs were prepared using the gentler method described in 4.8.2.

Table 6: The effect of convulsants on isoguvacine stimulated ^{36}Chloride flux in whole locust ganglia

	Isoguvacine stimulated ^{36}Cl- flux (cpm)	± s.d.	n
control	149	18	10
+ TBPS (23 μM)	93	8	6
+ Ptx (50 μM)	132	13	6

Using the Mann Whitney U test it was found that the data for TBPS were significantly different from control, whilst those for picrotoxinin were not.

Chapter 6 : Discussion

6.1 [^{35}S]TBPS Binding to Locust Ganglia P2 Membranes

6.1.1 Linearity of [^{35}S]TBPS binding with variation of P2 membrane concentration

In fig. 7 only the specific binding of [^{35}S]TBPS passes through the origin. Both total and non-specific bound [^{35}S]TBPS were 8 fmoles at 0 membrane concentration. This is a measure of the non-specific binding to non-membranous materials in the assay. It is probably mostly adsorption to the glass-fibre filters used to separate bound and free [^{35}S]TBPS.

6.1.2 pH dependence of [^{35}S]TBPS binding to locust ganglia membranes

Figure 8 shows that [^{35}S]TBPS binding to locust ganglia membranes has a pH optimum of 9. This is in reasonable agreement with the data reported by Squires *et al.* (1983) of 7.5-8.5 in rat brain membranes. Although it is greater than that reported by Szamraj *et al.* (1986), of 7.5 in house fly head membranes and by Ozoe *et al.* (1986), who reported a pH optimum of 7.5 for the binding of [^{3}H]propylbicyclicphosphate (a cage convulsant) to house fly head extracts.

As mentioned in Chapter 5, further studies were carried out at the sub-maximum pH of 7.4 in order to increase the assays ability to detect modulation of the cage convulsant site by other GABAergic ligands.

6.1.3 Chloride dependence of [^{35}S]TBPS binding to locust ganglia membranes

Figure 9 shows that the binding of [^{35}S]TBPS to locust ganglia membranes is enhanced by NaCl. However NaCl is not an absolute requirement as the line does not extrapolate to zero at zero NaCl. Squires et al. (1983) reported that [^{35}S]TBPS binding to rat brain membranes was entirely dependent on the presence of suitable halide ions. This was also noted by Abalis et al. (1985) for [^{35}S]TBPS binding to a putative voltage dependent chloride channel in torpedo electric organ. Whilst in invertebrate tissues Cohen & Casida (1986) have reported that the binding of [^{35}S]TBPS to house fly thorax and abdomen preparations was stimulated by NaCl upto 50 mM, but reduced at higher concentrations. They also noted that the presence of chloride ions was not an absolute requirement. More recently Olsen et al. (1988) have shown that [^{35}S]TBPS binding to house fly head membranes was enhanced by chloride ions up to 300 mM, although there was no absolute requirement for chloride ions. In contrast Ozoe et al. (1986) reported that the binding of [^{3}H]propyl bicyclic phosphate to house fly head extracts

was unaffected by NaCl (200 mM), whilst it was inhibited by 40% by 200 mM Na or KBr.

These observations and electrophysiological experiments on the anion permeability of GABA gated chloride channels have produced good correlations between the potencies of various anion's ability to enhance [^{35}S]TBPS binding and their permeabilities through GABA gated chloride channels. This has led to the hypothesis that anions increase [^{35}S]TBPS binding to GABA gated chloride channels by acting at specific anion binding sites that effect conformational changes in the receptor proteins (Marvizon & Skolnick, 1988). Such a specific anion binding site could explain the ability of Cl^- (& F^-) to produce bi-phasic (inhibition & enhancement) competition curves for GABA agonists and barbiturates, whereas only inhibition is seen in the presence of Br^- (Supavilai & Karobath, 1984 and Abel et al., 1987).

It is interesting to note that the invertebrate studies indicate that invertebrate GABA gated chloride channel may be slightly different from their vertebrate counterparts in not having an absolute requirement for halide anions.

6.1.4 Analysis of [^{35}S]TBPS binding to locust ganglia membranes

When the data for the specific binding of [^{35}S]TBPS over the range 2 - 150 nM were plotted as specific bound versus the ratio bound over free, (Scatchard analysis), the resultant line was curvelinear, concave downwards (fig. 11). This type of behaviour is indicative of more than one interacting site for the ligand and is strongly suggestive of positive cooperativity. Additionally when the Hill plot of the same data was examined (fig. 12), it too was non-linear.

De Lean & Rodbard (1979) have extended the general model of cooperative binding proposed by De Meyts & Roth (1975) into two models. One where k_{off} (k_{-1}) or dissociation of the ligand is a linear function of occupancy and the second where k_{on} (k_1) or association, is a linear function of occupancy.

The Scatchard and Hill analysese of the binding of [^{35}S]TBPS (2-150 nM) to locust ganglia membranes have a number of characteristics in common with each of the models proposed by De Lean & Rodbard, but both models are required to explain all the effects.

Model I: k_{off} as a function of occupancy

This predicts that for positive cooperativity the Scatchard plot will become horizontal, which is what occurs at higher concentrations of ligand. However it excludes the low-dose hook which is seen in fig. 11.

Additionally the Hill plot is not linear and concave upwards.

From this model, the Hill coefficient:

$$n_H = (\sqrt{d} + 1) / 2\sqrt{d}$$

where; d = interaction factor.

$$F_{0.5} = (d+1) / (2K_e)$$

where; $F_{0.5}$ = concentration of the ligand to give 0.5 occupancy of the receptors,

K_e = equilibrium constant of association at 0 occupancy.

From fig. 12 the $n_H = 2$ at the maximum deviation. Thus d = 0.11.

From computer-aided dose/response curve fitting of the data in fig. 14 the $F_{0.5}$ = 400 nM, thus K_e = 1.4 μM^{-1}

Additionally this model predicts that, with positive cooperativity, the dissociation of the labelled ligand initiated by larger concentrations of unlabelled ligand, when plotted as %bound versus time would give a curve concave upwards, whereas dissociation initiated by infinite dilution would give a linear response. This can be seen to be the case for [^{35}S]TBPS dissociation from locust ganglia membranes in fig. 19. The non-linear response when the dissociation is initiated by unlabelled TBPS can be seen to be due to re-association (fig. 21) of the radiolabelled TBPS. This could be due to the positive cooperative effects of the higher concentrations of unlabelled TBPS on the equilibrium position, overcoming the competitive effect.

Model II : k_{on} as a function of occupancy

In contrast to the first model this model predicts a low-dose hook seen in fig. 11 and is considered to be diagnostic of positive cooperativity. The low-dose hook response is most pronounced when both the labelled ligand and receptor concentrations are very low. A condition easy to achieve in locust due to the small amount of ganglionic tissue available from each locust. When the ligand and receptor concentrations are high > $1/K_e$ the hook tends to flatten out and disappear. Most workers set up their assay conditions such that the latter case is more likely and thus these interactions can be missed.

Figure 13 shows the Scatchard analysis for [^{35}S]TBPS binding to locust ganglia membranes over the range 10 - 50 nM and a 5 fold higher membrane concentration than used for the experiments shown in fig. 11. The linear response could be due to higher receptor concentrations. Although even at higher membrane concentrations the horizontal Scatchard was still most common.

Additionally this model predicts that for positive cooperativity, small concentrations of unlabelled ligand would have an anomalous positive effect on the association of the labelled ligand. This can be seen to be the case for [^{35}S]TBPS association to locust ganglia membranes in fig. 18. This effect can also be seen in

the equilibrium competition experiments for TBPS and IBP against [^{35}S]TBPS binding to locust ganglia membranes (fig. 22), where at lower concentrations the binding was greater than 100 %.

The low-dose hook, inhibition of dissociation by TBPS and enhancement of association can be simplistically explained by a configurational model:

In locust ganglia membranes there must be at least two binding sites for TBPS which are able to interact with each other through some physicochemical mechanism. The model does not need to define whether the binding sites are on the same polypeptide or on different polypeptides which form a multimer. In the absence of TBPS the sites are in a ground state (T) which has a given affinity for TBPS. When a molecule of TBPS binds to one of the sites, the interaction between the site and TBPS forces it into a new configuration (R). This new configuration can be passed on to the other site(s). If the R state has a higher affinity for TBPS then positive cooperativity will be the result. (The inverse will give negative cooperativity).

It is thus possible to envisage that when small amounts of labelled TBPS bind they cause a change in state of other TBPS binding sites and one gets the low-dose hook on the Scatchard analysis.

With the dissociation experiments, initiated by unlabelled TBPS, the initial effect is a competitive displacement. However the addition of unlabelled TBPS increases the total concentration of TBPS (unlabelled + labelled), this results in a further shift of sites to the R state, more TBPS occupies more binding sites, resulting in an increase in affinity for TBPS. This pushes the reaction towards a new equilibrium position and TBPS begins to re-associate. A very similar mechanism to the low-dose hook results in small concentrations of TBPS being able to enhance the association of [^{35}S]TBPS.

In figures 15 & 16 it was observed that the association of [^{35}S]TBPS to locust ganglia membranes was bi-phasic, with what appears to be an early (<60 min.) and late (>90 min.) phase. The early association constants varied with concentration of [^{35}S]TBPS at concentrations < 30 nM. Whilst the later association constants varied with concentrations > 40 nM (fig. 17).

This effect was also reported for [^{3}H]propyl bicyclophosphate binding to a house fly preparation (Ozoe et al. 1986).

Thus the analysis of data for [^{35}S]TBPS binding to locust ganglia membranes seems to indicate that:

a) Locust ganglia membranes have multiple, interacting binding sites for TBPS.

b) These sites seem to interact in a manner consistent with positive cooperativity brought about by changes in dissociation and association rates.

c) The affinity of these sites for TBPS is around 30 nM, (although the concentration which produces 50 % occupancy is around 400 nM).

d) The association of TBPS is bi-phasic, with an early stage more affected by lower concentrations of TBPS than the later stage.

e) The concentration dependence of the early and late stages of TBPS association coupled with the apparent saturation seen in early experiments at 50 nM (fig. 10a) may suggest the existence of additional low affinity sites for TBPS.

Chapter 6 : Discussion

The maximum [^{35}S]TBPS bound, (B_{max}) was approximately 1000 fmoles/mg of membrane protein, estimated by computer dose/response curve fitting. This is approximately twice the B_{max} reported for [^{3}H]flunitrazepam binding to locust ganglia membranes (Robinson et al., 1986) and suggests that there is a TBPS binding site on each of the polypeptides in the GABA receptor complex. (see 6.2.3). The level of GABAergic binding to locust ganglia membranes has been consistently found to be considerably lower than that for flunitrazepam (and thus TBPS), (Lunt et al., 1985 & Rutherford et al., 1987). This does not correlate with the probable alpha$_2$beta$_2$ (see 6.2.3) structure of the GABA receptor complex.

Of other studies in vertebrates and invertebrates only Ozoe (1987) has reported a non-linear Scatchard plot for the binding of [^{3}H]propyl bicyclophosphate to house fly head preparation. However this was concave upwards (indicating multiple sites or negative cooperativity). The Hill plot was linear with a Hill coefficient (n_H) approximately equal to unity. He interprets this as ruling out the possibility of cooperativity between the sites. He additionally reports that the dissociation was bi/poly-phasic as did Squires et al., (1983), and Maksay & Ticku (1985) for [^{35}S]TBPS

binding in rat brain, and Olsen et al. (1988) for [^{35}S]TBPS binding in house fly head membranes.

However Maksay & Ticku (1985) explain the poly-phasic dissociation of [^{35}S]TBPS as the result of endogenous GABA accelerating the dissociation and not as multiple binding sites. They found linear association and dissociation of [^{35}S]TBPS in extensively freeze/thawed membranes, but the dissociation became poly-phasic when GABA was re-introduced. They further propose that the effects of certain ligands on the kinetic acceleration of TBPS dissociation and the observed bi-phasic equilibrium effects (Supavilai & Karobath, 1984), suggest an analogy between an exposed state of the convulsant sites and an open state of the chloride ionophore. However they exclude any kinetic cooperativity between TBPS binding sites. This hypothesis will be further discussed in the next section.

Chapter 6 : Discussion

The reported affinities of binding sites for TBPS fall into a number of groups:

Vertebrates:

in the presence of bromide ions;

a) K_D= 20 nM (Squires et al., 1983)
(Ramanjaneyulu & Ticku, 1984)
(Supavilai & Karobath, 1984)
(Ticku & Ramanjaneyulu, 1984)

b) K_D= 1300 nM (Abalis et al.,1985)

in the presence of chloride ions;

c) K_D= 60 nM (Supavilai & Karobath, 1984)
= 50 nM (This thesis)
= 100 nM (Gallo et al., 1985)

Invertebrates:

in the presence of chloride ions;

d) K_D= 20 nM (Lummis & Sattelle, 1986)
= 50 nM (Olsen et al., 1988)
= 30 nM (This thesis)

no halide;

e) K_D= 210 nM (Cohen & Casida, 1986).

It appears that the affinities for TBPS show a similar trend to that seen for the effects of halide ions on the binding. Thus invertebrate tissues show a broad spread of affinities and do not have an absolute requirement for halides, whereas in the vertebrate tissues the affinities seem to be tightly grouped around values which vary with the halide (20 nM for Br^- and 60 nM for Cl^-). These tissues do have an absolute requirement for halide ions. Group (b) (Abalis et al., 1985) is the affinity of voltage dependent chloride channels for TBPS and is not GABA linked.

The binding of TBPS to locust ganglia membranes agrees with the other invertebrate reports. The affinity of the locust binding sites is about 30 nM. Although the concentration of TBPS required to give 50% saturation is about 400 nM, this is much less than that for the voltage dependent chloride channel binding site and very similar to the affinity reported by Cohen & Casida (1986) in house fly thorax and abdomen preparations.

6.1.5 Association rate constant for the binding of [^{35}S]TBPS binding to locust ganglia membranes

Although the association of TBPS to locust ganglia membranes appeared to have an early phase (<60 min.) and late phase (>90 min.), the approximate rate constants taken from the linear portions of the secondary plot (k_{1obs} versus concentration of [^{35}S]TBPS), were essentially the same, 0.23 & 0.26 fmoles/mg of membrane protein^{-1} min^{-1}. This indicates that a very similar mechanism is occurring for the association, but that it occurs at different times and over different concentration ranges of TBPS. This lends further support to the suggestion that there may be multiple sites for TBPS in locust ganglia membranes. Although the very fact that the derivative plots are non-linear indicate that there was more than association constant and that the kinetics were thus non-Michaelis-Menten. Therefore the above calculations are invalid.

6.1.6 Dissociation rate constant for the binding of [^{35}S]TBPS to locust ganglia membranes

Initial dissociation rate constants (k_{-1}) were estimated from the linear portions of the plots of $[B_t]/[B_0]$ versus time.

Dissociation initiated by excess unlabelled TBPS was quicker (28.6×10^{-3} min^{-1}) than that initiated by infinite dilution (9.5×10^{-3} min^{-1}).

Estimates of the K_D's can be made for each of these dissociation rate constants, using the formula:

$$K_D = k_{-1} / k_1$$

Thus using the value of 0.25 fmoles / mg of membrane $protein^{-1}$ min^{-1} for k_1:

K_D (TBPS) = 0.342 pM

K_D (dilution) = 0.114 pM,

where there was 0.3 mg protein(average) in a 100 µl assay.

These values indicate a substantially higher affinity for TBPS binding to locust ganglia membranes than the estimates from Scatchard and Hill plots. However as both association and dissociation experiments show deviations from the linear these values are invalid. The non-Michaelis-Menten kinetics indicated by association and dissociation experiments indicate that other constants are involved in the calculation of K_D, which the above, simple estimate has not accounted for. However it is

probably reasonable to expect that the estimates of the K_D from Scatchard and Hill plots are overestimates due to the cooperativity of the binding kinetics.

6.1.7 Competition by other ligands for the TBPS binding site in locust ganglia membranes

6.1.7.1 Convulsants

Only the two cage convulsants TBPS and IBP were found to displace the binding of [^{35}S]TBPS to locust ganglia membranes (fig. 22). The EC_{50}'s (TBPS, 0.68 μM and IBP 2.24 μM (table 4)) were obtained from Hill plots of the data in fig. 22. The value for TBPS is approximately 2 fold higher than that reported by Cohen & Casida (1986) for house fly thorax and abdomen preparations, but is 15 fold lower than the value reported by Olsen et al. (1988) for t-butylbicyclophosphate displacement of [^{35}S]TBPS binding to house fly head membranes, and the same as that for crayfish muscle membranes. Additionally the value for IBP is 10 fold lower than that reported by Olsen's group for house fly head but 2 fold higher than that for crayfish muscle.

It must be emphasised that the actual inhibition constant, (K_I) is dependent on the concentration of radioligand that the competing ligand has to displace

(Cheng & Prusoff, 1973). Thus for binding which shows Michaelis-Menten kinetics:

$$IC_{50}/[L] = K_I/K_D$$

where [L] = the concentration of radioligand.

This calculation makes no difference to the values reported by the groups of Cohen & Olsen, however the relationship is not valid for the binding of TBPS to locust ganglia membranes, as the previous two sections have demonstrated that the binding kinetics do not follow the rules of Michaelis-Menten. Thus comparisons should be treated with caution.

Picrotoxinin had no effect on the binding of TBPS to locust ganglia membranes at concentrations of 0.1 mM and below. This is in marked contrast to previous reports of TBPS binding in:

vertebrates: Squires & Casida, (1983) 190 nM; Gallo *et al*. (1985) 0.23 μM; Abalis *et al*. (1985) 100 μM; and

invertebrates: Cohen & Casida, (1986) 328 μM and Olsen *et al*. (1988) 15 μM. Values are IC_{50}'s or K_I's for picrotoxinin.

However locust ganglia membranes do contain binding sites for picrotoxinin because the pentobarbital enhancement of [^{3}H]GABA binding to locust ganglia membranes was totally abolished by 1 mM picrotoxinin (Rutherford et al., 1987). The significance of this and the inability of picrotoxinin to inhibit TBPS binding to locust ganglia membranes will be discussed in 6.1.7.5.

6.1.7.2 GABA

GABA was found to enhance the binding of TBPS to locust ganglia membranes (fig. 23), the maximum effect was 145% and the EC_{50} was 2.5 μM (table 4).

Squires et al., (1983) reported that GABA, muscimol and other GABA agonists were able to inhibit TBPS binding to rat brain in the presence of bromide ions, whilst in the presence of chloride ions, GABA had no effect. Supavilai & Karobath (1984) reported a biphasic action of muscimol in the presence of 200 mM NaCl, with a high affinity stimulation (EC_{50} 23 nM) and a low affinity inhibition (IC_{50} 724 nM), which corresponded to low and high affinity binding sites for [^{3}H]muscimol in the same preparation. Gallo et al. (1985) reported that [^{35}S]TBPS binding to cultured rat cerebellar neurons was only enhanced by muscimol, with the maximum effect in the range 1 - 5 μM muscimol.

Abalis et al. (1985) reported that binding of [^{35}S]TBPS to a putative voltage dependent chloride

channel was not affected by GABA. This and the fact that they could not demonstrate [^{3}H]muscimol or [^{3}H]flunitrazepam binding to the same preparation, led them to the conclusion that the site to which TBPS was binding was not part of a GABA receptor complex.

In the invertebrate studies, Cohen & Casida (1986) reported that [^{35}S]TBPS binding to house fly thorax and abdomen preparations was enhanced by GABA with an EC_{50} around 1 μM, which is in good agreement with that reported in this thesis for TBPS binding in locust ganglia membranes (table 4). However Lummis & Sattelle (1986) have reported that [^{35}S]TBPS binding to cockroach thoracic and abdominal ganglia was inhibited by GABA, but with a very similar potency, about 1 μM. More recently Olsen *et al.*, (1988) have reported that [^{35}S]TBPS binding to house fly head membranes was unaffected by GABA.

Unlike mammalian GABA binding studies, studies of GABA and GABA agonist binding to invertebrate tissues have failed to demonstrate (with the exception of Abalis & Eldefrawi (1986)) the existence of low affinity binding sites for GABA. However, as in mammals invertebrate neurons require high concentrations of GABA, mM or above (Beadle, 1988) before they respond in culture. Thus a high affinity receptor for GABA is not a necessity and

the binding studies may be detecting a down-regulated or otherwise inactivated receptor population.

Thus the concentrations of GABA which are able to enhance the binding of TBPS to locust ganglia membranes are well within the physiologically relevant range. One may deduce that the linkage between the GABA binding site and that for TBPS may have some physiological role and may well be involved with the gating mechanism of the chloride channel. Thus the TBPS binding site is part of a functional GABA receptor complex in locust ganglia membranes. The effect of GABA on the TBPS binding site in locust ganglia membranes provides a possible hypothesis to explain the cooperativity of TBPS binding to this tissue.

Electrophysiological studies with GABA (and other neurotransmitters) show sigmoid dose/response curves (response being ionic conductance). From such studies a model has been proposed where the ion channels can exist in two states, open or shut. Also it is proposed that the ligand receptor and the ion channel form a strongly interacting, membrane-localised complex, or 'protomer'. In concordance with the ion channel the protomer can exist in two states (corresponding to the open and closed states of the ion channel), for which the affinity of ligand molecules may be different. In the absence of any ligand the protomer adopts an equilibrium

between the T(closed) and R(open) states. In the presence of either agonist or antagonist the equilibrium can be perturbed between the two protomer states (Hollenberg 1985).

This is a more general description of Ehlert's two state model of the GABA receptor complex (2.2.4.2.1 & fig.5, Ehlert, 1986), which he used to explain the effects of agonist and inverse agonists at the benzodiazepine binding site. It is also highly analogous to the configurational model of cooperativity which was used to describe the kinetics of TBPS binding to locust ganglia membranes in the previous section. This fact and the sigmoid dose/response curves have given such models the title, 'cooperative receptor models' (Hollenberg ,1985).

Suppose the binding of TBPS was showing cooperative kinetics because of its integral role in a two state model for the GABA receptor complex, then one might expect that GABA and benzodiazepines would effect the cooperativity of TBPS binding to locust ganglia membranes. This is also suggested by the findings presented in this section (5.1.7), where the effects of GABA and Ro5-4864 on the TBPS inhibition of [^{35}S]TBPS were investigated.

A limited concentration range ($4x10^{-6}$ - 10^{-7} M TBPS) was selected to be on the borderline of the (cooperative)

deviation from the linear on the Hill plot. In the absence of any other ligand (except TBPS), this gave an estimate for the IC_{50} of 20 nM, (which agrees well with the estimates of the K_D at such points in binding experiments) and a Hill no. (n_H) = 1.4, indicating positive cooperativity. In the presence of 0.1 mM GABA the Hill no. fell to 1, indicating a lack of cooperativity over the range of concentrations which were cooperative in the control experiment. Conversely, Ro5-4864 at 0.1 mM, over the same concentration range, increased the Hill no. and decreased the IC_{50}. This suggests that the benzodiazepines enhance the cooperative effects of TBPS.

This agrees with a role for the convulsant binding site in Ehlert's two state model, in which benzodiazepines, in the absence of GABA, are only able to shift the equilibrium towards the open states, but GABA is able to push the equilibrium to the full open position. The fact that the action of TBPS is in the same direction as Ro5-4864 suggests that the cooperativity of TBPS binding shifts the GABA receptor complex towards the open state. This would not be consistent with its action as a non-competitive antagonist. However it is thought to act in an analogous manner to a 'plug'. Thus it is not inconceivable that forcing such a 'plug' (the action of increasing affinity by positive cooperativity) into a

closed channel may force the protomer into the open state, even though the channel lumen was still blocked by the 'plug'.

It is interesting to note that Maksay & Ticku (1985) suggested a connection between GABA's ability to accelerate the dissociation of TBPS binding from rat brain membranes and open and closed states of the GABA receptor complex, although they rule out any kinetic cooperativity of the TBPS binding site.

6.1.7.3 Benzodiazepines

The benzodiazepines were also able to enhance the binding of [^{35}S]TBPS to locust ganglia membranes (table 4). Interestingly the 'central' type benzodiazepine, clonazepam and the 'peripheral' type, Ro5-4864, appeared to have differential actions. Clonazepam had a 2 fold lower EC_{50} than Ro5-4864 but had a lower maximum effect (fig. 24). This is in contrast to previous studies of [^{3}H]flunitrazepam binding in locust ganglia membranes, (Robinson et al., 1986) and similar studies in cockroach ganglia membranes, (Lummis & Sattelle, 1986), where Ro5-4864 was more potent than clonazepam at displacing [^{3}H]flunitrazepam binding. However more recent studies of [^{3}H]Ro15-1788 binding to locust ganglia membranes (Rutherford et al., 1988) have indicated that clonazepam and Ro5-4864 were equipotent at enhancing the binding (same EC_{50}) although Ro5-4864 gave the largest maximum effect. It is interesting to note the possible bi-phasic shape of the curve for Ro5-4864, possibly indicating two sites, one of which clonazepam did not recognise. This would correlate with the mixed pharmacology of the locust benzodiazepine receptor described below.

In mammalian studies the effect of benzodiazepines on TBPS binding is no clearer. Wood et al. (1984) reported that the binding of [^{35}S]TBPS to rat brain membranes was enhanced, up to 150% by benzodiazepine

agonists whereas the inverse agonists produced a 20% reduction in binding. This work was repeated in cultured rat cerebellar neurons by Gallo et al. (1985). The effects of the agonists and inverse agonists on the binding of TBPS is consistent with their presumed effects in Ehlert's two state model of the GABA receptor complex. However Gee et al. (1986) report that the benzodiazepine agonists and inverse agonists have the opposite effect, (agonists inhibiting and inverse agonists enhancing), on [^{35}S]TBPS binding to rat cortex and hippocampus membranes. Although they still find that they have opposing effects to each other and are thus still consistent with Ehlert's two state model. The picture is further obscured by Ticku & Ramanjaneyulu's (1984) findings that Ro5-4864 was a competitive inhibitor of [^{35}S]TBPS binding to various brain regions of the rat, although more recently Gee et al. (1988) have reported that in rat brain membranes, Ro5-4864 was able to enhance [^{35}S]TBPS binding, by allosteric modulation, from a site within the GABA receptor complex, but which is distinct from the 'central' benzodiazepine site.

This work suggests further heterogenity of the benzodiazepine sites which are linked to the GABA receptor complex, in addition to those classified as 'peripheral' sites.

Chapter 6 : Discussion

A similar heterogenity of benzodiazepine binding sites, within the GABA receptor complex, has been inferred from a series of experiments on the locust ganglia benzodiazepine binding site.

Initial experiments with [^{3}H]flunitrazepam binding to locust ganglia membranes, indicated a mixed pharmacology between the, 'peripheral' and 'central' type, benzodiazepine receptors of mammalian CNS studies. Ro5-4864 was much more potent at displacing [^{3}H]flunitrazepam binding than clonazepam (Robinson *et al.*, 1986), (a peripheral characteristic). However GABA and its agonist, isoguvacine were able to enhance the binding of [^{3}H]flunitrazepam (Robinson *et al.*, 1986). This suggests, that the binding site in locust ganglia membranes was part of a GABA receptor complex. In mammals, only 'central' type benzodiazepine receptors are thought to be linked to the GABA receptor complex. However more recently, Gee *et al.* (1988) have reported the existence of a binding site for Ro5-4864, which is part of the GABA receptor complex, but is not the 'central' benzodiazepine binding site.

Additionally Robinson *et al.* (1986) were able to photoaffinity label membranes from the ganglia of the locust using [^{3}H]flunitrazepam, (another characteristic of the central benzodiazepine sites in mammalian studies (Thomas & Tallman, 1981)). The photoaffinity labelling

was also enhanced by GABA. However flunitrazepam was able to photoaffinity label two polypeptides (M_r 45K & 59K). Whilst in parallel experiments in rat brain membranes [^{3}H]flunitrazepam only photoaffinity labelled one polypeptide (M_r 48.5K), which is in agreement with that reported by Thomas & Tallman (1981).

More recently further photoaffinity labelling studies have been carried out on locust ganglia membranes using Ro15-4513. This central type ligand, partial inverse agonist, only photoaffinity labels the central benzodiazepine sites in mammalian brain (Mohler et al., 1984). In locust ganglia membranes this ligand labelled only one polypeptide, (M_r 57.5K) (Riley, personal communication) approximately the same size as the larger polypeptide labelled by flunitrazepam. This result led to the hypothesis that the mixed pharmacology of the benzodiazepine binding site in locust ganglia membranes could be due to the presence in GABA receptor complexes, of both peripheral and central type benzodiazepine binding sites (either separately or even within the same complex). However it was also found that the photoaffinity labelling of locust ganglia membranes by [^{3}H]Ro15-4513 was more potently inhibited by Ro5-4864 than by clonazepam (Riley, personal comunication). Thus even the supposed 'central' binding sites in locust have some peripheral characteristics.

Further complication has been added by the results of [^{3}H]Ro15-1788 (a benzodiazepine antagonist) binding to locust ganglia membranes. This ligand binds to locust ganglia membranes with higher affinity than flunitrazepam, but there are approximately 0.5-0.6 the number of sites (B_{max}). Additionally when the binding is displaced by unlabelled flunitrazepam instead of Ro15-1788, a bell-shaped Scatchard curve is obtained, concave downwards; such behaviour is indicative of positive cooperativity. However in this case it appears to be between the binding site for Ro15-1788 and that for flunitrazepam. All the benzodiazepines tested were able to enhance Ro15-1788 binding, Ro5-4864 and clonazepam were equipotent (Rutherford et al. 1988).

Although the heterogenity of benzodiazepine binding sites in vertebrate and invertebrate CNS is far from a simple explanation, it does appear that there are similarities with both sets of problems and one solution may yet be found (see 6.2.3).

However, what can be concluded from these previous reports is, that the enhancement of [^{35}S]TBPS binding by Ro5-4864 and clonazepam, in locust ganglia membranes, is further evidence that this binding site is part of a GABA receptor complex.

6.1.7.4 Barbiturates

Pentobarbital was the only barbiturate available to be assayed. Pentobarbital was also able to enhance the binding of [^{35}S]TBPS to locust ganglia membranes (fig.25 & table 4). Pentobarbital was the most potent enhancing ligand tested (smallest EC_{50}), although it did not give as large a maximum effect as dieldrin (table 4). Currently interest in the the barbiturate site has been stimulated by observations that some steroids can modulate GABA in a manner similar to that of the barbiturates. Thus further studies using steroids, on the locust GABA receptor complex may yield interesting results.

Barbiturates have been proposed to be closely associated with the convulsant site of the GABA receptor complex as a result of early studies using [^{3}H]dihydropicrotoxinin as a probe for the site. Ticku & Olsen (1978) proposed from such a study that the depressant and convulsant barbiturates act via the picrotoxinin sensitive site in the GABA receptor complex. However convulsant barbiturates gave Hill numbers <1, indicating, either multiple binding sites or negative cooperativity, although they discounted multiple sites as unlabelled dihydropicrotoxinin was able to displace the labelled with a Hill no. = 1.

Subsequently with the introduction of TBPS as a probe for the convulsant binding site Squires et al., (1983), found that the inhibition of [^{35}S]TBPS binding by barbiturates was comparable to that for [^{3}H]dihydropicrotoxinin (IC_{50}, pentobarbital = 100 μM). However the next year Supavilai & Karobath (1984) reported a biphasic effect of pentobarbital on [^{35}S]TBPS binding to rat brain, with enhancement at lower concentrations (EC_{50} 60.4 μM) and inhibition at higher concentrations (IC_{50} 550 μM). The Hill no. for the inhibition was 2.7, indicating multiple, positively interacting sites. Trifiletti et al (1984) reported that barbiturates inhibit [^{35}S]TBPS binding in a mixed competitive manner and also accelerate the dissociation of TBPS, which, they suggest, indicates that the barbiturate and TBPS binding sites are separate but allosterically linked.

Thus the potent enhancing effect of pentobarbital on the binding of [^{35}S]TBPS to locust ganglia membranes is probably also through an allosteric modulation from another binding site within the GABA receptor complex. However the small EC_{50} suggests that this site is very tightly linked to that for TBPS. There is the possibility that this effect is mediated through another convulsant site (see 6.1.7.5).

6.1.7.5 Insecticides

In section 2.3.1.2 the relationship between certain groups of insecticides and the convulsant binding site of the GABA receptor complex was first discussed. It was Ghiasuddin & Matsumura (1982), working with GABA stimulated chloride flux into cockroach nerves, who first introduced the idea that a cyclodiene, (heptachlor epoxide)and lindane could antagonise the stimulatory effect of GABA. Subsequent binding experiments with [^{35}S]TBPS were done in rat brain and indicated that, cyclodienes, lindane (Lawrence & Casida, 1984) and type II pyrethroids (Lawrence & Casida, 1983) were potent competitive inhibitors of TBPS binding.

However when [^{35}S]TBPS binding in locust ganglia membranes was studied, these groups of insecticides were found to be potent enhancers of TBPS binding.

Dieldrin (a cyclodiene) was the most potent (smallest EC_{50} = 1.4 μM and largest maximum response = 310%), whilst lindane was only half as potent as dieldrin (EC_{50}= 2.5 μM, twice as big and maximum effect = 175%, half as big) (table 4). These values are the same as those reported for the inhibition of [^{35}S]TBPS to rat brain by, dieldrin (IC_{50} = 1.4 μM) and lindane (IC_{50} = 1.7 μM) (Lawrence & Casida, 1984). However lindane gave different effects in different experiments, in 6 experiments it showed a shallow inhibition (IC_{50} = 10

μM), which only reached 50% inhibition before higher concentrations became enhancing (fig. 26). This was very similar to the behaviour of picrotoxinin (data not shown) which showed 320% enhancement of TBPS binding at 0.1 M which fell to 35% inhibition at 0.01 M, but by 0.1 mM was back to 100%. However the concentrations of ethanol required to keep these concentrations of picrotoxinin in solution make the results subject to extremely large errors consequently they have not been shown.

Cypermethrin (a type II pyrethroid) was also able to enhance the binding of [^{35}S]TBPS to locust ganglia membranes, whilst permethrin (cypermethrin with hydrogens instead of the alpha-cyano group, therefore a type I pyrethroid) was without effect on the binding of TBPS. This is in agreement with Lawrence & Casida, (1983), who found that only type II pyrethroids were able to inhibit [^{35}S]TBPS binding to rat brain membranes but the enhancement in locust is a significant difference. Additionally, Crofton et al. (1987) reported that the K_I for cypermethrin inhibition of [^{35}S]TBPS binding to rat brain was 7.6 μM, which is 6 fold lower than the EC_{50} for the cypermethrin enhancement of [^{35}S]TBPS binding in locust ganglia membranes (table 4).

These results in the locust contrast with those in the house fly, Cohen & Casida (1986) have found that [^{35}S]TBPS binding to house fly thorax and abdomen preparations was potently inhibited by various cyclodienes (IC_{50} for dieldrin = 0.8 μM). More recently Olsen et al (1988) have reported that [^{35}S]TBPS binding to house fly head membranes was also potently inhibited by cyclodienes (IC_{50} for dieldrin = 0.05 μM) and lindane (IC_{50} = 0.2 μM). However they only report small inhibitions by various isomers of cypermethrin.

The reasons for these differences between locust and mammals, and locust and house fly are not clear. It may well be connected with the high interaction between the binding sites in the locust GABA receptor complex which lead to the positive cooperativity in the binding kinetics for TBPS. It is also important to remember that the house fly studies have been carried out in gross tissue preparations (whole head and whole thorax & abdomen), which almost certainly contain digestive enzymes and other contaminants. This should be considered when the house fly studies are compared with this study on a cleaner tissue preparation, the supraoesophageal ganglion of the locust.

The enhancing effects of these insecticides on TBPS binding in locust suggest that they do not bind to the same site as TBPS, but to an allosterically linked site within the GABA receptor complex. However it has been shown that the groups of insecticides which can antagonise GABA neurotransmission are unable to antagonise either [^{3}H]muscimol or [^{3}H]flunitrazepam binding (Eldefrawi *et al.*, 1985). The most consistent report concerns the action of these insecticides on the picrotoxinin binding site, recently reviewed by Matsumura *et al.*, (1987). Although initial work has suggested that picrotoxinin and the cage convulsants bind at the same site More recent work by Casida with the cage convulsants may challenge that hypothesis.

The initial challenge came from the observation that picrotoxinin has some insecticidal action, whilst TBPS has little or none (Casida, 1988). Casida's group have synthesised trioxabicyclooctanes with much bigger substituents. These compounds are much more insecticidal than the smaller trioxabicyclooctanes, such as TBPS. 4-*s*-[^{3}H]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane is such a compound which has been radiolabelled by Casida's group, to enable ligand binding studies to be carried out. Such studies in the CNS of the American cockroach have produced some very

interesting results. Whilst the binding of this compound was inhibited by picrotoxinin and the polychlorocycloalkane insecticides, such as dieldrin and lindane, it was not inhibited by TBPS (Nicholson et al., 1988a). Casida has explained these data by proposing that there may be two sub-groups of cage convulsant: Group A, with longer substituents, are most active in insects. Group A binding sites may be the site of action of polychlorocycloalkane insecticides and picrotoxinin. Group B, have the smaller substituents (eg TBPS), and are least active in insects, but extremely potent in mammals.

Thus in the locust it is possible there are 2 binding sites for convulsants: a group B site, which has been characterized using [^{35}S]TBPS; and a group A site, which is allosterically linked and is the site of action of picrotoxinin and the insecticides studied. The lack of effect of picrotoxinin could be explained by it having a lower affinity for the group A site than the insecticides. This would correlate with the uncertain effects seen at very high picrotoxinin concentrations. Additionally there is evidence that a picrotoxinin binding site does exist in the locust GABA receptor complex, as picrotoxinin is able to block the barbiturate enhancement of GABA binding (Rutherford et al., 1987).

6.2 Binding of [^{35}S]TBPS to Rat brain membranes

The binding of [^{35}S]TBPS to locust ganglia membranes, as described in the previous sections, is significantly different from that reported for the binding of the same ligand to rat brain membranes. The significant differences are: the cooperative binding kinetics, the lack of effect of the convulsant, picrotoxinin and the enhancement by GABA.

In order to discount the possibility that these differences resulted from to a systematic error in the methodology used for the experiments a parallel study was done on the binding of TBPS to rat brain membranes.

6.2.1 The binding parameters of [^{35}S]TBPS binding to rat brain membranes

Figure 27 shows the binding isotherm for [^{35}S]TBPS binding in rat brain membranes, it approximates very well to a rectangular hyperbola and is not sigmoid. The Hill plot of these data gave a Hill coefficient = 1, indicating a single, non-interacting population of binding sites. Computer dose/response curve fitting, estimated the K_D to be about 50 nM, which agrees very well with previous assays of [^{35}S]TBPS binding to rat brain membranes in the presence of chloride ions (Supavilai & Karobath, 1984).

6.2.2 The effect of picrotoxinin & GABA on the binding of [^{35}S]TBPS binding to rat brain membranes

Both picrotoxinin and GABA were able to inhibit the binding of [^{35}S]TBPS to rat brain membranes (figs. 28 & 29). The IC_{50} for picrotoxinin is similar to the values reported by Squires et al., (1983), and Ramanjaneyulu & Ticku (1984); it is important to remember that these workers carried out their assay in the presence of bromide ions. It has been previously noted that the different halide ions have a profound effect on the pharmacology of the convulsant binding site (Supavilai & Karobath, 1984; Abel et al.,1987). However the IC_{50} for GABA was significantly larger than that previously reported for rat brain membranes. This could also be a cumulative effect of the halide ion, as Squires et al. (1983) have reported a very strong effect of the concentration of GABA, on the enhancement of [^{35}S]TBPS binding by various halide anions.

It is evident from these results that methodological error can be ruled out as a cause of the differences observed in the previous sections.

6.2.3 Differences between mammalian & locust convulsant binding sites

Why does the locust convulsant binding site display cooperative binding kinetics for TBPS, which the rat does not show? In 6.1.3, De Lean & Rodbards second model of cooperativity, (where k_{on} was a function of occupancy), it was noted that the low-dose hook effect on the Scatchard plot was most pronounced when both the labelled ligand and receptor concentrations are very low. Most ligand binding assays on mammalian brain tissue are set up so as to avoid such a condition and thus the low-dose hook effect can be absent from the Scatchard. Thus it is possible that, due to the low levels of tissue available from the locust, this study has highlighted an interaction that has been overlooked in mammalian studies. It is already well established that the various ligands which have binding sites on the GABA receptor are able to allosterically modulate each other (Olsen, 1981), and this behaviour can be included in Ehlert's two-state model for the GABA receptor complex (fig.5 Ehlert, 1986), which is a 'cooperative receptor model' for the GABA receptor complex. Essentially all the various allosteric interactions, between various ligand binding sites, result in stabilising either the open or closed state of the chloride ion channel. A reasonable hypothesis would be

that ligands which bind to, or near the ion channel, such as the cage convulsants may be sensitive to whether the channel was in an open or closed state. A logical follow-on to such a hypothesis would be to suggest that such sensitivity could be exhibited as cooperative binding of the cage convulsants.

An alternative hypothesis is a possible result of the molecular architecture of the GABA receptor complex, which has recently been fully sequenced by the groups of Barnard & Seeburg (Schofield et al., 1987). Two polypeptides have been sequenced, corresponding to the two sub-units of the GABA receptor, alpha and beta (Mohler et al., 1987). RNA's were made from these two sequences and expressed in the Xenopus oocyte, only when the two sequences were injected together was any response to GABA found in the oocytes. The effect of GABA on the oocytes was potently blocked by picrotoxinin (Schofield et al., 1987). These results indicate that only the two sub-units (alpha & beta) are required to give a functional GABA receptor. Thus the chloride channel is a composite structure formed by the two sub-units. Current theories propose that the GABA receptor complex is a $alpha_2beta_2$ multimer (Schofield et al., 1987). Thus as the channel is a composite of four sub-units it seems reasonable to propose that the cage convulsant site may exist as a composite site or in four (or two, if it is on only one type of sub-unit) copies. The GABA binding site resides on the beta sub-unit and the benzodiazepine binding site on the alpha (Mohler et al., 1987). There is much evidence that these sites, on distinct polypeptides, can allosterically modulate each other (Olsen, 1981). Therefore one could propose that the convulsant site, a composite or in multiple copies,

could also be involved in these allosteric modulations and thus display cooperative binding kinetics. This hypothesis offers an alternative explanation to that based on receptor concentration for the differences between rat and locust TBPS binding. Hebebrand et al. (1988) have proposed the existence of 'isoreceptors', (by analogy to isozymes), to explain subtle differences in pharmacology in different brain regions, for the nicotinic acetylcholine and GABA receptors, both members of a proposed 'super-family' of ligand gated ion channels (Schofield et al., 1987). He proposes that differences in benzodiazepine pharmacology and the regional distribution of such differences could be explained by the presence of additional alpha sub-units which have a high degree of homology, but differ slightly in their affinity for certain ligands. This could also affect the convulsant binding site as it must be part of one or both of the sub-units. One would expect that there would be larger differences between isoreceptors of different species, due to their evolutionary separation. This could be an explanation of the observed differences between rat and locust and even locust and house fly TBPS binding.

6.3 ^{36}Chloride Flux into Locust ganglia preparations

In the last section it was inferred that there might be two cage convulsant binding sites in the locust, a group A site sensitive to picrotoxinin and the polychlorocycloalkane insecticides and a group B site, sensitive to TBPS. However binding studies as, described in the previous sections, give no information as to the connections between the convulsant binding site and the chloride channel. How might these two cage convulsant binding sites be linked to the chloride channel? Are they linked to the chloride channel? Such questions cannot be answered definitively by binding studies and a more physiological approach needs to be taken. In the absence of access to electrophysiological equipment, it was decided to attempt the approach introduced by Harris & Allan, (1985) in rat brain and more recently in cockroach nerve cords (Wafford et al., 1987), in which the movement of $^{36}Cl^-$ into or out of membranous vesicles prepared from nervous tissue is measured.

6.3.1 ^{36}chloride flux into microsacs prepared using the method of Wafford et al. (1987)

Initial experiments using this method were encouraging (fig. 30). GABA stimulation of $^{36}Cl^-$ flux increased with incubation time and with increasing concentration of GABA and was reduced to control levels in the presence of 0.1 mM Triton X100. These results suggested that microsacs prepared from locust ganglia were accumulating $^{36}Cl^-$ in a time dependent (fig 30b) manner which was associated with GABA function and with intact vesicles. However the long preparation time seemed to introduce a variability into the preparations such that often no signal was obtained at at all. Large errors were also observed in the cockroach nerve cord microsacs described by Wafford et al., (1987). Such variability made the investigation of the effects of other ligands on the GABA stimulated $^{36}Cl^-$ flux difficult. A technique had previously been developed in our laboratory for the rapid production, (in one step), of locust ganglia synaptosomes (Robinson, 1986). It was hoped that the rapidity of this technique would avoid the variability found with that of Wafford et al., (1987).

6.3.2 ^{36}chloride flux into microsacs prepared using the method of Robinson (1986)

Using this preparation, GABA stimulated $^{36}Cl^-$ flux, reaches a maximum in 20s instead of about 60s with the preparation of Wafford et al. ^{36}Chloride was also stimulated by the GABA agonists, isoguvacine and APS (table 5). In agreement with GABA (& GABA agonist) binding studies in invertebrate tissues, bicuculline was unable to block the effect of GABA (Lunt et al., 1985; Abalis & Eldefrawi, 1986; Lummis & Sattelle, 1986; Rutherford et al., 1987). However the convulsants, picrotoxinin and TBPS were also unable to block the GABA stimulated $^{36}Cl^-$ flux. The only compound which was able to block the effect of GABA on the chloride flux was the chloride channel blocker, DIDS. The IC_{50} for DIDS inhibition of GABA stimulated Cl^- flux was 4 μM (fig. 32), which is smaller than that reported by Abalis et al., (1986) for Cl^- flux into rat brain microsacs. The EC_{50} for GABA in this preparation was 0.8 μM (fig. 33). This value is approximately the same as that for GABA on cockroach nerve cord microsacs (Wafford et al., 1987), but lower than that reported for rat brain microsacs, EC_{50}=15 μM (Abalis et al., 1986) and whole cockroach nerve cords EC_{50}=80 μM (Ghiasuddin & Matsumura, 1981). Osmotic shock (addition of distilled water to the assay), reduced the $^{36}Cl^-$ flux to below control levels.

This indicates that some form of vesicular preparation is involved in these effects.

The lack of effect of both picrotoxinin and TBPS was disturbing in view of the fact that intact vesicles were present and that DIDS was able to block the GABA stimulated $^{36}Cl^-$ flux. In studies of structure/toxicity relationships in mice, the high toxicity of the cage convulsants has been attributed to three structural features: their caged shape and symmetry, a hydrophobic substituent of critical volume and the presence of a strong overall molecular dipole (Casida et al., 1976). This has led to the hypothesis that the cage convulsants interact with a pore-type receptor, fitting into the lumen of the pore in such a way as to block its normal physiological function (Ozoe & Eto, 1986). Thus their non-competitive antagonist action (Bowery et al., 1976) is thought to be through a channel blocking mechanism. Is this the case in the locust?

6.3.3 ^{36}Chloride flux into whole locust ganglia

To test if the convulsants were able to act as channel blockers in the locust assays of $^{36}Cl^-$ flux were carried out in whole ganglia, using a technique modified from that described by Ghiasuddin & Matsumura, (1982). By not preparing the ganglia in any way it was hoped that some action of the convulsants could be detected which might have been lost during the previous preparations. During solubilisation studies of the mammalian GABA receptor complex it was the convulsant site which was the most labile of all the binding sites (Stephenson, 1988).

It is interesting to note that in the time course of $^{36}Cl^-$ flux in whole ganglia (fig. 34), even the control shows considerable chloride flux. This is in contrast to the case with both microsac preparations, where the control chloride flux stayed at a stable basal rate. This effect in whole ganglia could be due to the presence of intact neuronal circuits giving rise to the release of GABA. The longer time course for whole ganglia compared to microsacs is in agreement with previous data for whole tissues (Ghiasuddin & Matsumura, 1982).

In whole ganglia, TBPS was able to inhibit the isoguvacine stimulated $^{36}Cl^-$ flux, but picrotoxinin did not. This is in contrast to electrophysiological experiments with cultured locust neurons where picrotoxinin was a weak channel blocker (Beadle, 1988), but agrees with the actions of these two convulsants in the previous binding studies and suggests that the TBPS binding site might be more integral with the chloride channel than the picrotoxinin binding site (6.3.4).

Isoguvacine was used for these studies as it would be stable against GABA uptake and metabolising enzymes. The higher concentrations were used to ensure sufficient agonist was able to diffuse into the whole tissue.

Summary

The experiments described above are very preliminary, however a number of points may be made:

1) In microsac and whole ganglia preparations of the locust, one is able to demonstrate GABA and agonist stimulated $^{36}Cl^-$ flux.

2) Detergent and osmotic shock can stop such effects, indicating that intact vesicles are required.

3) The effects of GABA can be blocked by the chloride channel blocker DIDS and TBPS, but not picrotoxinin.

However there are severe limitations to the further development of such experiments:

a) The lack of a reproducible method of producing stable insect synaptosomes/microsacs etc.

b) The low $^{36}Cl^-$ flux signals make the visualization of the effect of a ligand very difficult.

Both these limitations need to be overcome before one will be able to do more than the type of very simple experiments described above. Nevertheless such experiments have provided some interesting answers (and questions), which make improving these experiments an important task for the future.

Chapter 7

7.1 Conclusions

The findings for the binding of [^{35}S]TBPS to locust ganglia membranes, are consistent with the presence in locust ganglia of a specific binding site for TBPS which do not show Michaelis-Menten kinetics. However estimates put the K_D at approximately 30 nM, but the $F_{0.5}$ is approximately 400 nM. The B_{max} is at most 1200 fmoles/mg of membrane protein.

There is suggestive evidence that the binding site shows positive cooperativity as a result of changes in both on and off rates with occupation by TBPS. This may well be a result of the binding site's integral role in a GABA receptor complex.

The enhancement of the binding by GABA, benzodiazepines and pentobarbital is further evidence that the binding site for TBPS is part of a GABA receptor complex, similar to that described in mammals.

The chloride enhancement of the binding, is consistent with a close association between the binding site and the chloride channel of the GABA receptor complex. In agreement with other invertebrate studies there appears to be no absolute requirement for chloride, this is in contrast with previous vertebrate studies.

The lack of effect of picrotoxinin on the binding of TBPS, but its ability to block pentobarbital enhancement of GABA binding, suggests the presence of a second convulsant site. This is also suggested by the enhancement of TBPS binding by insecticides which have been previously shown to give 'picrotoxinin-like' poisoning. The existence of 2 convulsant binding sites is a logical extension of Casida's hypothesis that the cage convulsants can be divided into two groups: group A, including picrotoxinin; and group B, including TBPS.

The demonstration of Michaelis-Menten binding kinetics for TBPS, in parallel experiments in rat brain membranes, and the inhibition of this binding by GABA and picrotoxinin, indicate that the results in locust ganglia membranes, were not due to systematic methodological error in the experiments. The differences between rat and locust TBPS binding may be due the membrane concentration, but it may well prove to be due to the existence of different isoreceptors of the TBPS binding protein between rat and locust. Only when the locust GABA receptor complex has been fully sequenced will the reasons begin to be understood.

Initial experiments to demonstrate GABAergic chloride flux in various locust ganglia preparations are encouraging. Although it seems progress will be very slow until a reproducible method of producing 'membrane

vesicles' is found for invertebrate tissues. First indications are that TBPS may be a better channel blocker than picrotoxinin. However due to the problems of reproducibility further investigation of this effect is necessary.

7.2 Overview

This thesis contains a description of the initial work to characterise the cage convulsant binding site of the GABA receptor complex, in the CNS of the Desert Locust. Like previous studies on the GABA receptor complex in locust (Lunt et al., 1985 & Robinson et al., 1986), the general conclusion is that the locust GABA receptor complex contains a similar collection of binding sites to that found in mammalian studies. However this and previous studies have indicated that there are differences in the pharmacology and the way in which the sites are linked together.

Due to the association of the cage convulsant site with the mode of action of various insecticides in mammalian studies, the study of this binding site in insect species has aroused special interest. It is interesting that the cage convulsant binding site in locust differs, in its cooperative TBPS binding kinetics and in its interaction with the insecticides mentioned above, from that described in rat brain. In addition the locust cage convulsant differs from that in house fly

(Cohen & Casida, 1986 & Olsen et al., 1988), although as mentioned above, it is difficult to compare the studies on crude preparations from the house fly, with this study on isolated ganglia from the locust. However these differences between invertebrate and vertebrate species and possibly between invertebrate species, offer hope that through continued effort to characterise such insecticide target sites, one may be able to develop insect specific and even species specific insecticides. This would be a large step towards the ideal pesticide (2.3.4).

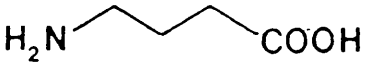
As mentioned in the previous chapter, not only is our knowledge of chemical neurotransmission in insects, behind that in mammals, but also our knowledge of the molecular structure of the target sites under study. Only recently has work increased in this field; this will be of great importance to the understanding of the neurochemistry which is now being carried out.

APPENDIX I

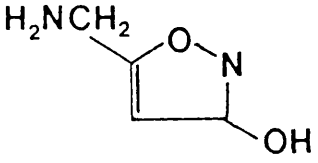
Structures of the Ligands used

GABA, Agonists & Antagonists

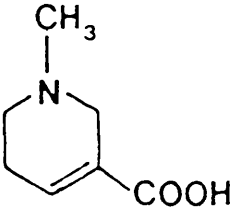
GABA



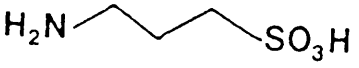
muscimol



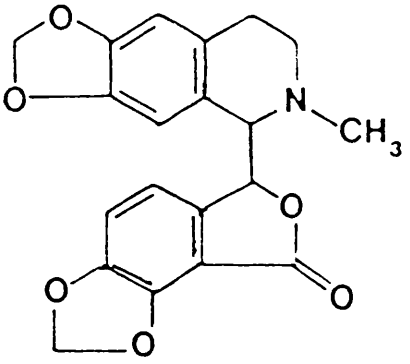
isoguvacine



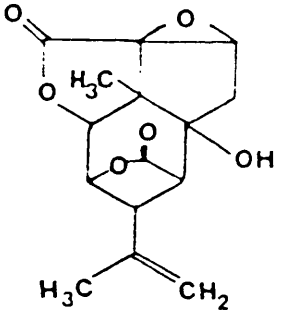
3APS



bicuculline

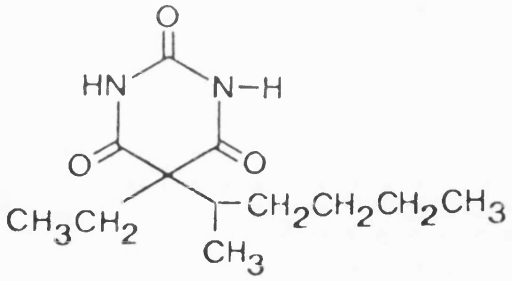


picrotoxinin

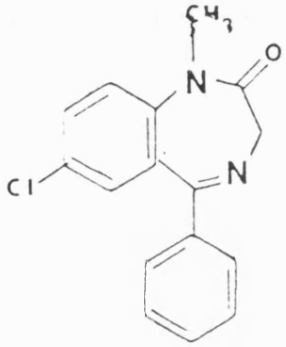


Barbiturate & Benzodiazepines

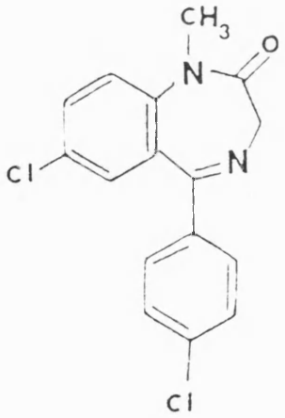
pentobarbital



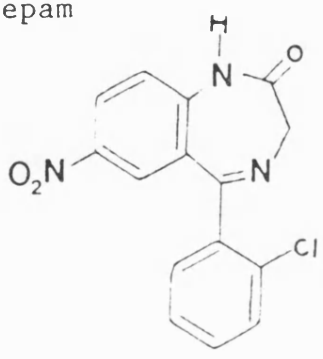
diazepam



Ro5-4864

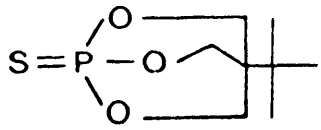


clonazepam

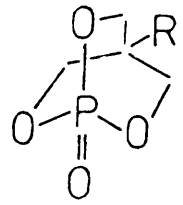


Cage Convulsants

TBPS



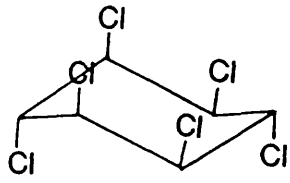
IBP



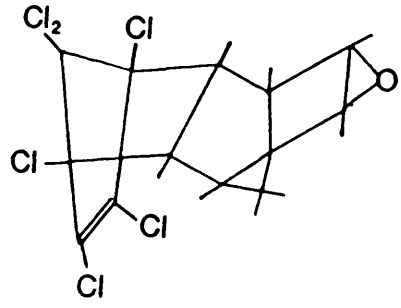
R = isopropyl

Insecticides

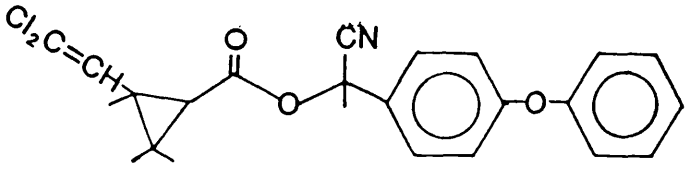
lindane



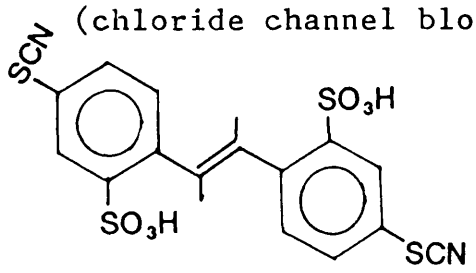
dieldrin



cypermethrin



DIDS (chloride channel blocker)



APPENDIX II

Derivation of the equations to calculate the rate constants

Dissociation rate constant, k_{-1}

In general, the rate of change of the concentration of bound ligand is the difference between the rates of association of the ligand and the receptor and of dissociation of the ligand/receptor complex (RL), as stated in equation(eg)1:

$$d(RL)/dt = k_1(L)(R)-k_{-1}(RL) \qquad \text{eq1.}$$

At equilibrium $d(RL)/dt = 0$.

If the experimental conditions are such that the rate of association of ligand (L) and receptor (R) is negligible, then the measured rate of change of bound ligand is the dissociation rate of RL:

$$d(RL)/dt = -k_{-1}(RL) \qquad \text{eq2.}$$

Experimentally the association of R and L is made to be negligible by 'infinitely' diluting a mixture of R and L which has been allowed to come to equilibrium, or by adding a large excess of unlabelled ligand.

Integration and rearrangement of eq2 gives:

$$\ln[(RL)/(RL)_0] = -k_{-1}t \qquad \text{eq3,}$$

in which $(RL)_0$ is the concentration of radioligand bound at time = 0, just prior to the dilution or addition of excess unlabelled ligand.

Thus the negative slope of a plot of $\ln(RL)/(RL)_0$ versus time, provides an estimate of the dissociation rate constant, k_{-1}.

As RL = the amount of specific bound radioligand (B), eq3 can be re-written:

$$\ln (B / B_0) = -k_{-1}t$$

as used in 4.3.

Association rate constant, k_1

From eq1, one can see that, if the concentration of radioligand is much higher than the total concentration of receptors in the incubation, then, as the reaction proceeds, the concentration of free ligand (L) does not change appreciably, while the concentration of unoccupied receptors (R), does decrease significantly. Hence for all practical purposes (L) is constant throughout the duration of the reaction and a

new constant $k_1' = k_1(L)$ can be defined. Thus, the reaction is considered to be a 'pseudo-first order' reaction. Substitution of k_1' into eq1, gives:

$$d(RL)/dt = k_1'(R)-k_{-1}(RL) \qquad \text{eq4,}$$

The following substitutions for R can be made:

$$R = \begin{cases} R_t-RL_{eqm} \\ RL_{eqm}+R_{eqm}-RL \\ RL_{eqm}+RL_{eqm}(k_{-1}/k_1')-RL \end{cases}$$

and eq4 becomes:

$$dRL/dt = (k_1'+k_{-1})(RL_{eqm}-RL) \qquad \text{eq5,}$$

integration of this equation gives:

$$RL = RL_{eqm}(1-e^{-(k_1'+k_{-1})t}) \qquad \text{eq6,}$$

which can be rearranged in the form:

$$\ln[(RL_{eqm})/(RL_{eqm}-RL)] = (k_1'+k_{-1})t \qquad \text{eq7.}$$

Equation 7 can be used to compute the association rate constant k_1 from experimental binding data. At time = 0, the radioligand at concentration (L) is added to a suspension of receptors. The amount bound (B = RL) is measured at various times until the equilibrium level of binding ($B_{eqm} = RL_{eqm}$) is reached. Then, the term $\ln B_{eqm} / (B_{eqm} - B)$ is plotted against time. The slope of the line is the experimentally observed apparent rate constant, $k_{1observed}$:

$$k_{1obs} = k_1' + k_{-1},$$

or

$$= k_1(L) + k_{-1} \qquad \text{eq8.}$$

If k_{1obs} is determined at several concentrations of L, a plot of k_{1obs} versus (L) has a slope of k_1 and an intercept with the ordinate of k_{-1}.

For further details see Morris (1974).

APPENDIX III

Sections of the work presented in this thesis have previously been published and are listed below.

Brown,M.C.S, Lunt,G.G. & Stapleton,A. (1987) t-Butylbicyclophosphoro[^{35}]thionate binding to ganglionic membranes of the locust Schistocerca gregaria. Biochem. Soc. Trans. 15(3), 503-504.

Brown,M.C.S., Lunt,G.G. & Stapleton,A. (1987) The cage convulsant site of the locust GABA receptor complex differs from its counterpart in mammalian brain. Abstr. Neurosci. 13, part 2, 961.

Brown,M.C.S., Lunt,G.G. & Stapleton,A. Differences betweeen the [^{35}S]TBPS binding site in locust ganglia membranes and that in rat brain. Biochem. Soc. Trans. (in press)

Brown,M.C.S., Lunt,G.G. & Stapleton,A. Further characterisation of the [^{35}S]TBPS binding site in locust ganglia membranes. Comp. Biochem. Physiol. (in press)

Brown,M.C.S., Lunt,G.G. & Stapleton,A. Investigation of chloride flux in membrane vesicles prepared from the supraoesophageal ganglia of the locust, Schistocerca gregaria. Pestici. Sci. (in press)

REFERENCES

Abalis,I.M. & Eldefrawi,A.T. (1986) [^{3}H]muscimol binding to a putative GABA receptor in honeybee brain and its interactions with avermectin B1a. Pest. Biochem. Physiol. 25, 279-87.

Abalis,I.M., Eldefrawi,A.T. & Eldefrawi,M.E. (1986) Actions of avermectin B1a on the gamma-aminobutyric acid-A receptor and chloride channels in rat brain. J. Biochem. Toxicol. 1, 69-82.

Abalis,I.M., Eldefrawi,M.E. & Eldefrawi,A.T. (1983) Biochemical identification of putative GABA/benzodiazepine receptors in housefly thorax muscles. Pest. Biochem. Physiol. 20, 39-48.

Abalis,I.M., Eldefrawi,M.E. & Eldefrawi,A.T. (1985) Binding of GABA receptor channel drugs to a putative voltage-dependent chloride channel in Torpedo Electric Organ. Biochem. Pharmacol. 34(14), 2579-2582.

Abel,M.S., Garrett,K.M. & Blume,A.J. (1987) Differential effects of halide ions on allosteric interactions of the $GABA_A$ receptor complex. Abstr. 17th ASN 13(2) 961.

Ackerman,D. & Kutsher,F. (1910) Uber aporrhegmen. Z. Physiol. Chem. 69, 1265-72.

Alberquerque,E.X., Alkondon,M., Deshpande,S.S., Cintra,W.M. & Brossi,A. (1988) The role of carbamates and oximes in reversing toxicity of organophosphorous

compounds: a perspective into mechanisms. Proceedings of Neurotox '88, Nottingham. in press).

Awapara,J., Landua,A.J., Fuerst,R. & Searle,B. (1950) Free gamma-aminobutyric acid in brain. J. Biol. Chem. 187, 35-9.

Baron,S. (1972) The Desert Locust. Eyre Metheun, London.

Beadle,D.J. (1988) Neuronal cultures as experimental systems. In; Comparative invertebrate neurochemistry (Lunt,G.G. & Olsen,R.W., eds.) pp. 227-255. Croom Helm, London & Sydney

Beadle,D.J., Benson,J.A., Lees,G. & Neuman,R. (1985) Flunitrazepam and pentobarbital modulate GABA responses of insect neuronal somata. J. Physiol. 2731, 273.

Bonetti,E.P., Pieri,L., Cumin,R., Schaffner,R., Pieri,M., Gamzu,E.R., Muller,R.K.M. & Haefely,W. (1982) Benzodiazepine antagonist Ro15-1788: Neurological and behavioural effects. Psychopharmacology 78, 8-18.

Bowery,N.G., Collins,J.F. & Hill,R.G. (1976) Bicyclic phosphorous esters that are potent convulsants and GABA antagonists. Nature 261, 601-3.

Bradford,H.F. (1986) Chemical Neurobiology. pp. 229-42. W.H. Freeman & Co., New York.

Braestrup,C., Schmiechen,R., Neef,G., Nielsen,M. & Petersen,E.N. (1982) Interaction of convulsive ligands with benzodiazepine receptors. Science 216, 1241-43.

Breer,H. & Heilgenberg,H. (1986) Neurochemistry of GABAergic activities in the central nervous system of Locusta migratoria. J. Comp. Physiol. A 157, 343-54.

Breer,H. & Jeserich,G. (1980) A microscale flotation technique for the isolation of synaptosomes from the nervous tissue of Locusta migratoria. Insect Biochem. 10, 457-63.

Burg,R.W., Miller,B.M., Baker,E.E., Birnbaum,J., Currie,S.A.Hartman,R., Kong,Y.L., Monaghan,R.L., Olson,R. & Omura,S. (1979) Avermectins, new family of potent anthelmintic agents: Producing organism and fermentation. Antimicrob. Agents Chemther. 15, 361-7.

Butts,W.L. & Davidson,R.H. (1955) The toxicity of five organic insecticides to Blatella germanica. J. Econ. Entomol. 48, 572-4.

Calcott,P.H. & Fatig,R.O. (1984) Avermectin modulation of GABA binding to membranes of rat brain, brine shrimp and a fungus, Mucor miehei. J. Antibiotics XXXVII(7), 797-801.

Callec,J.J. (1974) Synaptic transmission in the central nervous system of insects. In; Insect neurobiology. (Treherne,J.E., ed.) pp. 119-78. Elsevier North-Holland, Amsterdam.

Casida,J.E., Eto,M., Moscioni,A.D., Engel,J.L., Milbrath,D.S. & Verkade,J.G. (1976) Structure-toxicity

relationships of 2,6,7-trioxabicyclo[2.2.2]-octanes and related compounds. Toxicol. App. Pharmacol. 36, 261-279.

Casida,J.E., Nicholson,R.A. & Palmer,C.J. (1988) Trioxabicyclooctanes as probes for the convulsant site of the GABA-gated chloride channel. Pestici. Sci. (in press).

Chapman,R.F. (1975) The insects structure & function. The English Universities Press Ltd. London.

Cheng,Y. & Prusoff,W.H. (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 % inhibition (I_{50}) of an enzymatic reaction Biochem. Pharmacol. 22, 3099-3108.

Cohen,E. & Casida,J.E. (1986) Effects of insecticides and GABAergic agents on a house fly [^{35}S]-butylbicyclophosphorthionate binding site. Pestic. Biochem. Physiol. 25, 63-72.

Corbett,J.R. (1974) The Biochemical Mode of Action of Pesticides. Academic Press, London & New York.

Crofton,K.M., Reiter,L.W. & Mailman,R.B. (1987) Pyrethroid insecticides & radioligand displacement from the GABA receptor chloride ionophore complex. Toxicol. Letts. 35, 183-190.

De Lean,A. & Rodbard,D. (1979) Chapter4: Kinetics of cooperative binding. In; The Receptors Vol.1 General Principles and Procedures. (O'Brien, ed.) pp. 143-190 Plenum Press, New York & London.

De Meyts,P. & Roth,J. (1975) Cooperativity in ligand binding: A new graphic analysis. Biochem. Biophys. Res. Commun. 66, 1118.

Egerton,J.R., Ostinlind,D.A., Blair,L.S., Eary,C.H., Suhayda,D., Cifelli,S., Riek,R.F. & Campbell,W.C. (1979) Avermectins, new family of potent antihelminitic agents: Efficacy of the B1a component. Antimicrob. Agents Chemther. 15, 372-8.

Ehlert,F.J. (1986) 'Inverse agonists', cooperativity and drug action at benzodiazepine receptors. TIPS Jan.86, 28-32.

Eldefrawi,M.E., Sherby,S.M., Abalis,I.M. & Eldefrawi,A.T. (1985) Interactions of pryrethroid & cyclodiene insecticides with nicotinic acetycholine & GABA receptors. Neurotoxicol. 6(2), 47-62.

Ellis,P.E. (1959) Learning and social aggregation in locust hoppers. Anim. Behav. 7, 91-106.

Enna,S.J. & Snyder,S.H. (1975) Properties of gamma-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. Brain Res. 100, 81-97.

Gallo,V., Wise,B.C., Vaccarino,F. & Guidotti,A. (1985) Gamma-aminobutyric acid and benzodiazepine-induced modulation of [^{35}S]-t-butylbicyclophosphorothionate binding to cerebellar granule cells J. Neurosci. 5(9), 2432-8.

Gee,K.W., Brinton,R.E. & McEwen,B.S. (1988) Regional distribution of a Ro5-4864 binding site that is functionally coupled to the gamma-aminobutyric acid / benzodiazepine receptor complex in rat brain. J. Pharamacol. Exp. Ther. 244(1), 379-83.

Gee,K.W., Lawrence,L.J. & Yamamura,H.I. (1986) Modulation of the chloride ionophore by benzodiazepine receptor ligands: influence of gamma-aminobutyric acid and ligand efficacy. Mol. Pharamacol. 30(3), 218-25.

Gerschenfeld,H.M. (1973) Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. Phys. Rev. 53, 1-119.

Ghiasuddin,S.M. & Matsumura,F. (1982) Inhibition of gamma-aminobutyric acid (GABA)-induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp. Biochem. Physiol. 73C(1), 141-4.

Gordon,D., Zlotkin,E. & Kanner,B. (1982) Functional membrane vesicles from the nervous system of insects, 1. Na+ and Cl- dependent GABA transport. Biochim. Biophys. Acta 688, 229-36.

Grayson,J.McD. (1954) Differences between a resistant and a non-resistant strain of the German cockroach. J. Econ. Entomol. 47, 253-6.

Greenlee,D.V., Van Ness,P.C. & Olsen,R.W. (1978) Endogenous inhibitor of GABA binding in mammalian brain. Life Sci. 22, 1653-62.

Guidotti,A., Toffano,G., & Costa,E. (1978) An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain. Nature 275, 553-555.

Gunn,D.L. (1952) Field tests of dry baiting against the Desert Locust. Bull. ent. Res. 42, 675-690.

Gunn,D.L. (1960) Nomad encompassed. J. ent. Soc. S. Afr. 23, 65-125.

Gunn,D.L. (1979) Stratergies, systems, value judgements and dieldrin in control of locust hoppers. Phil. Trans. R. Soc. B287, 429-445.

Harris,R.A. & Allan,A.M. (1985) Functional coupling of gamma-aminobutyric acid receptors to chloride channels in brain membranes. Science 228, 1108-10.

Hebebrand,J., Friedl,W. & Propping,P. (1988) The concept of isoreceptors: application to the nicotinic acetylcholine receptor and the gamma-aminobutyric $acid_A$ / benzodiazepine receptor complex. J. Neural. Transm. 71, 1-9.

Hemming,C.F., Popov,G.B., Roffey,J. & Waloff,Z. (1979) Charateristics of Desert Locust plague upsurges. Phil. Trans. R. Soc. B287, 375-386.

Hill,A.V. (1910) The possible effects of the aggregation of the molecules of Haemoglobin on its dissociation curves. J. Physiol. 40, 190.

Hollenberg,M.D. (1985) Receptor models and the action of neurotransmitters and hormones: some new perspectives. In; Neurotransmitter receptor binding (Yamamura,H.I., Enna, S.J., Kuhar,M.J., eds.) 2nd edn. pp. 2-35. Raven Press, New York.

Humrich,A. & Richardson,A. (1983) 'By Hand' curve-fitting program for the analysis of multiple site radioligand binding data. Pharmacology 80, 582.

Iverson,L.L. & Kravitz,E.A. (1966) Uptake of gamma-aminobutyric acid (GABA) in a lobster nerve -muscle preparation. Fed. Proc. 25, 714.

Jacobson,M. & Crosby,D.G. (Eds.) (1971) Naturally Occuring Insecticides. Dekker, New York.

Jeffery,D., Rutherford,D.H., Weitzman,P.D.J. & Lunt,G.G. (1988) Purification & partial characterisation of 4-aminobutyrate :2oxoglutarate aminotransferase from sheep brain & locust ganglia. Biochem.J. 249(3) 795-799.

Kravitz,E.A. (1967) Acetylcholine, gamma-aminobutyric acid and glutamic acid: physiological and chemical studies related to their role as neurtransmitter agents. In; The neurosciences : a study program. (Quarton,G.C., Melnechuk,T. & Schmitt,F.O.,eds.) pp.433-44. Rockefeller University Press, New York.

Lawrence,L.J. & Casida,J.E. (1983) Stereospecific action pyrethroid insecticides on the gamma- aminobutyric acid receptor-ionophore complex. Science 221, 1399-1401.

Lawrence,L.J. & Casida,J.E. (1984) Interactions of lindane, toxaphene and cyclodienes with brain-specific t-butylbicycophosphorothionate receptor. Life Sci. 35(2), 171-8.

Lowery,O.H., Roseburgh,N.J., Farr,A.L. & Randall,R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lummis,S.C.R. & Sattelle,D.B. (1985a) Insect central nervous system gamma-aminobutyric acid. Neurosci. Lett. 60, 13-18.

Lummis,S.C.R. & Sattelle,D.B. (1985b) GABA and benzodiazepine binding sites in insect CNS. Pest. Sci. 16, 534.

Lummis,S.C.R. & Sattelle,D.B. (1986) Binding sites for [^{3}H]GABA, [^{3}H]flunitrazepam & [^{35}S]TBPS ininsect CNS Neurochem. Int. 9(2), 287-93.

Lunt,G.G., Robinson,T.N., Miller,T., Knowles,W.P. & Olsen R.W. (1985) The identification of GABA receptor binding sites in insect ganglia. Neurochem. Int. 7, 751-4.

Maksay,G., & Ticku,M.K. (1985) GABA, depressants and chloride ions affect the rate of dissociation of ^{35}S-t-butylbicyclophosphorothionate binding Life Sci. 37(23), 2173-80.

Mallamaire,A. & Roy,J. (1958) La lutte contre le criquet pelerin en Afrique Occidentalle Francaise. Bull. Prot. veg. 113.

Martin,H. (1973) The Scientific Principles of Crop Protection. 6th Edition. Arnold, London.

Marvizon,J.C.G. & Skolnick,P. (1988) Enhancement of t-[^{35}S]butylbicyclophosphorothionate and [3H]strychnine binding by monovalent anions reveals similaritiesbetween gamma-aminobutyric acid and glycine gated chloride channels J. Neurochem. 50(5), 1632-1639.

Matsumura,F. & Ghiasuddin,S.M. (1983) Evidence for similarities between cyclodiene type insecticides and picrotoxinin in their action mechanisms. J. Environ. Sci. Health B18(1), 1-14.

Matsumura,F., Tanaka,K. & Ozoe,Y. (1987) GABA-related systems as targets for insecticides. ACS Symposium : Sites of action for neurotoxic pesticides. (356), 44-70.

Meiners,B.M., Kehoe,P., Shaner,D.M. & Olsen,R.W. (1979) GABA receptor binding and uptake in membrane fractions of crayfish muscle. J. Neurochem. 32, 979-90.

Mohler,H., Battersby,M.K. & Richards,J.G. (1980) Benzodiazepine receptor protein identified and visualised in brain tissue by photoaffinity labelling. Proc. Natl. Acad. Sci. USA 77, 1666-70.

Mohler,H., Schoch,P., Richards,J.G., Haring,P. & Takacs,B. (1987) Structure and location of a GABA-A

receptor complex in the central nervous system. J. Receptor Res. 7(1-4), 617-28.

Mohler,H., Sieghart,W., Richards,J.G. & Hunkeler,W. (1984) Photoaffinity labeling of benzodiazepine receptors with a partial inverse agonist. Europ. J. Pharmacol. 102, 191-2.

Morris,J.G. (1974) Chapter 10; The kinetics of chemical reactions. In: A biologists physical chemistry. (Barrington,E.J.W. & Willis,A.J., eds.) Edward Arnold, London.

Moses (c1290 B.C.) Exodus The Holy Bible Chap. 10 verses 13-16.

Nicholson,R.A., Palmer,C.J. & Casida,J.E. (1988a) 4-s-[^{3}H]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]: specific binding in the American cockroach central nervous system. Pestici. Sci. (in press).

Nicholson,R.A., Robinson,P.S., Palmer,C.J. & Casida,J.E. (1988b) Ivermectin-stimulated release of neurotransmitter in the insect central nervous system: modulation by external chloride and inhibition by a novel trioxabicyclooctane and two polychlorocycloalkane insecticides. Pestici. Sci. (in press).

Nielsen,M., Braestrup,C. & Squires,R.F. (1978) Evidence for a late evolutionary appearance of brain- specific benzodiazepine receptors: an investigation of 18

vertebrate and 5 invertebrate species. Brain Res. 141, 342-6.

Nistri,A. Constanti,A. & Krnjevic,K. (1980) Electrophysiological studies of the mode of action of GABA on vertebrate central neurons. In; Advances in Biochemical Psychopharmacology, Vol.21: Receptors for Neurotransmitters and Peptide Hormones (Pepeu,G., Kuhar,M.J. & Enna,S.J., eds.) pp. 537-550. Elsevier, New York.

Olsen,R.W. (1980) Biochemistry of GABA receptor proteins in mammalian brain. In; Psychopharmacology and Biochemistry of Neurotransmitter Receptors (Yamamura,H.I., Olsen,R.W. & Usdin,E., eds.) pp. 537-50. Elsevier, New York.

Olsen,R.W. (1981) GABA-Benzodiazepine-Barbiturate receptor interactions. J. Neurochem. 37(1), 1-13.

Olsen,R.W. & Snowman,A.M. (1985) Avermectin B1a modulation of gamma-aminobutyric acid / benzodiazepine receptor binding in mammalian brain. J. Neurochem. 44(4), 1074-82.

Olsen,R.W., Szamraj,O. & Miller,T. [^{35}S]t-butylbicyclophosphorothionate (TBPS) binding sites ininvertebrate tissues J. Neurochem (submitted)

Olsen,R.W. ,Ticku,M.K. & Miller,T. (1978a) Dihydropicrotoxinin binding to crayfish muscle sites

possibly related to gamma-aminobutyric acid receptor -ionophores. Mol. Pharmacol. 14, 381-390.

Olsen,R.W. ,Ticku,M.K., Van Ness,P.C. & Greenlee,D. (1978b) Effects of drugs on gamma-aminobutyric acid receptors, uptake, release and synthesis in vitro. Brain Res. 139, 227-294.

Ozoe,Y. (1987) Bicyclic phosphorus esters: Insecticidal properties and binding sites in the housefly ACS Symposium Series 356, 83-96.

Ozoe,Y. & Eto,M. (1986) Bridged bicyclic organophosphorous compounds as a probe for toxicological study on GABA synapse. In; Membrane receptors and enzymes as targets of insecticidal action (Clark,J.M., ed) pp. 75-105.

Ozoe,Y., Eto,M., Mochida,K. & Nakamura,T. (1986) Characterisation of high affinity binding of [^{3}H]propyl bicyclic phosphate to house fly head extracts Pestic. Biochem. Physiol. 26, 263-274.

Pichon,Y. (1974) The pharmacology of the insect nervous system. In; The physiolgy of insects. (Rockstein,M., ed.) pp. 102-74. Academic Press, New York.

Pong,S.S. & Wang,C.C. (1982) Avermectin B1a, modulation of gamma-aminobutyric acid receptors in rat brain membranes. J. Neurochem. 38, 375-9.

Ramanjaneyulu,R. & Ticku,M.K. (1984) Binding characteristics and interactions of depressant drugs

with [^{35}S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. J. Neurochem. 42(1), 221-9.

Rao,Y.R. (1942) Some results of studies on the Desert Locust in India. Bull. ent. Res. 33, 241-265.

Regan,J.W., Yamamura,H.I., Yamada,S. & Roeske,W.R. (1981) High affinity renal [^{3}H]flunitrazepam binding: characterisation, localisation and alteration in hypertension. Life Sci. 28, 991-998.

Roberts,E. & Frankel,S. (1950) Gamma-aminobutyric acid in the brain; its formation from glutamic acid. J. Biol. Chem. 187, 55-63.

Robinson,T.N. (1986) GABA mediated neurotransmission in insects. Ph.D thesis, Bath University.

Robinson,T.N. & Lunt,G.G. (1986) A rapid technique for the production of locust synaptosomal preparation. Proc. 17th FEBS Meeting biol. Chem Hoppe Seiler, 367, suppl. 298.

Robinson,T.N. & Olsen,R.W. (1988) GABA In; Comparative Invertebrate Neurochemistry, (Lunt,G.G. & Olsen,R.W., eds.) pp. 90-123. Croom Helm, London & Sydney.

Robinson,T.N. ,MacAllan,D., Lunt,G.G. & Battersby,M. (1986) Gamma-aminobutyric acid receptor complex of insect CNS: Characterisation of a benzodiazepine binding site. J. Neurochem. 47(6), 1955-62.

Rutherford,D.M., Jeffery,D., Lunt,G.G. & Weitzman,P.D.J. (1987) GABA binding to receptor sites in locust supraoesophageal ganglia Neuroscience Lett. 79, 337-40.

Rutherford,D.M., Jeffery,D., Lunt,G.G. & Weitzman,P.D.J. (1988) Benzodiazepine receptors in locust (Schistocerca gregaria): binding of Ro15-1788 Biochem. Soc. Trans. 16(3), 310.

Sayer,H.J. (1959) An ultra-low-volume spraying technique for the control of the Desert Locust. Bull. ent. Res. 50, 371- 386.

Scatchard,G. (1949) The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci. 51, 660-672.

Schaeffer,J.M., Haines,H.W. & Turner,M.J. (1988) Avermectin binding in Caenorhabditis elegans: a two state model for the avermectin binding site. Neurotox 88 Abstr. pp. 107-8.

Schoemaker,H., Bliss,M. & Yamamura,H.I. (1981) Specific high-affinity saturable binding of [^{3}H]Ro5-4864 to benzodiazepine binding sites in rat cerebral cortex. Eur. J. Pharmacol. 71, 173-175.

Schofield,P.R., Darlison,M.G., Fujita,N., Burt,D.R., Stephenson,F.A., Rodriguez,H., Rhee,L.M., Ramachandran,J., Reale,V., Glencourse,T.A., Seeburg,P.H. & Barnard,E.A. (1987) Sequence and functional expression

of the GABA-A receptor shows a ligand gated receptor super-family. Nature 328, 221-27.

Shankland,D.L. & Schroeder,M.E. (1973) Pharmacological evidence for a discrete neurotoxic action of dieldrin in the American cockroach, Periplaneta americana. Pestic. Biochem. Physiol. 3, 77-86.

Squires,R.F., Casida,J.E., Richardson,M,. & Saederup,E. (1983) [^{35}S]t-Butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to gamma-amino- butyric acid-A & ion recognition sites. Mol. Pharmacol. 23, 326-336.

Stapleton,A.G. (1986) Studies on L-glutamic acid decarboxylase from locust brain. Ph.D. Thesis. UMIST, England.

Stephenson,F.A. (1988) Understanding the $GABA_A$ receptor: a chemically gated ion channel. Biochem. J. 249, 21-32.

Study,R.E. & Barker,J.L. (1981) Diazepam & (-)-pentobarbital: Fluctuation analysis reveals different mechanisms for potentiation of gamma-aminobutyric acid responses in cultured neurons. Proc. Natl. Acad. Sci. USA 78, 7180-7184.

Supavilai,P. & Karobath,M. (1984) [^{35}S]-tert-Butylbicyclophosphorothionate binding sites are constituents of the gamma-aminobutyric acid benzodiazepine receptor complex. J. Neurosci. 4(5), 1193-200.

Supavilai,P., Mannonen,A. & Karobath,M. (1982) Modulation of GABA binding sites by CNS depressants and CNS convulsants. Neurochem. Int. 4, 259-268.

Szamraj,O.I., Miller,T. & Olsen,R.W. (1986) Cage convulsant [35S]TBPS binding to GABA receptor-chloride channel complex in invertebrate tissues Abst. Soc. Neurosci. 12, 656.

Tallman,J.F., Paul,S.M., Skolnick,P. & Gallager,D.W. (1980) Receptors for the age of anxiety: Pharmacology of the benzodiazepines. Science 207, 274-281.

Tanaka,K. & Matsumura,F. (1985) Action of avermectin B1a on the leg muscles and the nervous system of the American cockroach. Pest. Biochem. Physiol. 29, 124-35.

Tanaka,K., Scott,J.G. & Matsumura,F. (1984) Picrotoxin receptor in the central nervous system of the American cockroach: its role in the action of cyclodiene insecticides. Pest. Biochem. Physiol. 22, 117-27.

The Anti-Locust Research Centre (1966) The Locust Handbook. Ministry of Overseas Development, London. Chapter 8.

Thomas,J.W. & Tallman,J.F. (1981) Characterisation of photoaffinity labelling of benzodiazep-ine binding sites. J. Biol. Chem. 256, 9838-42.

Ticku,M.K. & Olsen,R.W. (1978) Interaction of barbiturates with dihydropicrotoxinin binding sites

related to the GABA receptor-ionophore system. Life Sci. 22, 1643-1652.

Ticku,M.K. & Olsen,R.W. (1979) Cage convulsants inhibit picrotoxinin binding. Neuropharmacology 18, 315-8.

Ticku,M.K. & Ramanjaneyulu,R. (1984) Ro5-4864 inhibits the binding of [^{35}S]t-butylbicyclophosphorothionate to rat brain membranes. Life Sci. 34, 631-638.

Trifiletti,R.R., Snowman,A.M. & Snyder,S.H. (1984) Barbiturate recognition site on the GABA/benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site. Eur. J. Pharmacol. 106(2), 441-7.

Turner,A.J. & Whittle,S.R. (1983) Biochemical dissection of the gamma-aminobutyrate synapse. Biochem. J. 209, 29-41.

Udenfriend,S. (1950) Identification of gamma-aminobutyric acid in brain by the isotope derivative method. J. Biol. Chem. 187, 33-59.

Uvarov,B.P. (1921) A revision of the genus Locusta, with a new theory as to the periodicity and migrations of locusts. Bull. ent. Res. 12, 135-163.

Wafford,K.A., Sattelle,D.B., Abalis,I., Eldefrawi,A.T. and Eldefrawi,M.E. (1987) GABA activated ^{36}chloride influx: A functional in vitro assay for CNS GABA receptors of insects. J. Neurochem. 48(1), 177-180.

Waloff,Z. (1966) The upsurges and recessions of the Desert Locust plague: an Historical survey. Anti-Locust Mem. 8.

Waloff,Z. (1972) The plague dynamics of the Desert Locust, Schistocerca gregaria. In Proceedings of the International Study on the current and Future problems of Acridology, London. (Hemming,C.F. and Taylor,T.H.C., eds.) 343-349.

Williams,M. & Risley,E.A. (1979) Characterisation of the binding of [^{3}H]muscimol, a potent gamma-aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. J. Neurochem. 32, 713-18.

Wood,P.L., Loo,P., Braunwalder,A., Yokoyama,N. & Cheney,D.L.(1984) In vitro characterisation of benzodiazepine receptor agonist-s, inverse agonists and agonist/antagonists. J. Pharmacol. Exp. Ther. 231(3), 572-6.